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(54) Title: TREATING RED BLOOD CELL SOLUTIONS WITH ANTI-VIRAL AGENTS			
(57) Abstract <p>Methods and compositions for treating pathogens in material are described, including methods of decontaminating human fluids prior to processing in the clinical laboratory and methods for decontaminating blood products prior to <i>in vivo</i> use. The techniques handle large volumes of human blood products without destroying the function of the blood products. Novel compounds for photodecontaminating biological material are also contemplated which are compatible with clinical testing, in that they do not interfere with serum analytes.</p>			

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TREATING RED BLOOD CELL SOLUTIONS WITH ANTI-VIRAL AGENTS

This application is a continuation in part of U.S. Patent Application Serial
5 No. 08/338,040, filed November 14, 1994.

FIELD OF THE INVENTION

The invention generally relates to new compounds and methods for the
in *vitro* inactivation of pathogens in biological material intended for *in vitro* or
10 *in vivo* use, and in particular the inactivation of pathogens in solutions
containing red blood cells, prior to clinical testing or transfusion.

BACKGROUND

15 The presence of pathogens in blood products, as well as other biological
materials, is recognized as a significant health problem to health workers as well
as recipients of the materials.

With regard to health workers, a great volume of human fluids is handled
daily as part of the routine monitoring of hospital patients by obtaining and
testing human fluids (blood, urine, spinal fluid, etc.). Typically, each admitted
20 patient has at least a tube of blood collected every day by a phlebotomist. During
the transferring, portioning and testing process, each sample tube is handled by a
clinical worker while its contents are exposed. This intensive handling of
potentially infectious human fluids is not without health risk. The Occupational
Safety and Health Administration (OSHA) estimates that over five million
25 health workers, including hospital laboratory workers, are exposed to blood
borne-pathogen infections in the work place annually. The pathogen responsible
for the overwhelming majority of infections is the hepatitis B virus (HBV). The
Center for Disease Control (CDC) estimates there are twelve thousand cases of
HBV infection among health workers each year. Of these cases, over five
30 hundred require hospitalization and approximately two hundred and fifty of
these patients die (i.e. from fulminant hepatitis, cirrhosis or liver cancer). See
Guidelines for Prevention of Transmission of HIV and HBV to Health-Care and
Public Safety Workers, CDC (February 1989). Most full time laboratory employees
contract hepatitis at least once during their career. Indeed, up to one third of all
35 health care workers show serological evidence of a previous HBV infection. Id.

Following the recognition of Acquired Immunodeficiency Syndrome (AIDS), clinical laboratories have instituted additional precautions. For example, rather than using manually positioned plastic inserts to maintain the separation of cells from serum after samples are centrifuged, a "gel" is now available that is 5 in the empty tube at the time the blood is drawn. When the tube is centrifuged the cells go below the gel while the serum remains above. While the separation can be maintained in this manner without as much sample handling, this does not reduce the handling of the technologist at the point of analysis. Unfortunately, an infectious virus can persist in a liquid or dried state for 10 prolonged periods of time, possibly even at elevated temperatures. Resnick *et al.*, JAMA 255:1887 (1986).

Preventative measures such as gloves and eye-wear are not complete solutions to the problem. Accidents in the laboratory or clinic typically involve exposure over a larger portion of the body and disease can be transmitted 15 through the skin and mucous membranes. Morbidity and Mortality Weekly Report 36:285 (1987).

Clearly, there remains a need for a more adequate solution to blood borne-pathogen infections in the work place. Such a solution should serve as a protection against a wide range of pathogens. Furthermore, the mechanics of the 20 solution should not unduly interfere with operations of a laboratory or blood bank.

Another significant problem is the contamination of the blood supply for *in vivo* use. The safety of the blood supply continues to be threatened by the transmission of pathogens by transfusion. While the threat posed by the human 25 immunodeficiency virus (HIV) and the Acquired Immune Deficiency Syndrome (AIDS) is now widely publicized, contamination of blood products with a number of other blood-borne infectious viral agents is of even greater concern. See R. Y. Dodd, In: Transfusion Medicine in the 1990's (American Assoc. Blood Banks 1990) (S.J. Nance, ed.). For example, in the United States, it is estimated 30 that up to ten (10) percent of multiply transfused recipients develop hepatitis accounting for many thousands of cases annually.

Whole blood collected from volunteer donors for transfusion recipients is typically separated into its components: red blood cells, platelets, and plasma. Each of these fractions are individually stored and used to treat a multiplicity of 35 specific conditions and disease states.

The red blood cell component is used primarily to treat trauma, chronic anemia, and blood loss due to surgery (particularly cardiac and liver surgery), including postoperative bleeding. D.M. Surgenor *et al.* Transfusion 32:458 (1992). Approximately twelve (12) million units of red cells are transfused into 5 approximately four (4) million recipients annually in the United States alone. E.L. Wallace *et al.* Transfusion 33:139 (1993).

The safety of the blood supply cannot be assured by merely testing the blood for pathogens before transfusion. Most testing relies on the detection of antibodies to the pathogen in the prospective blood donor. It is now well-documented that infectious agents can be transmitted by "seronegative" blood 10 donors, i.e. donors that have no detectable antibodies to the pathogen. For example, thirteen cases of transfusion-related AIDS have been reported to the Centers for Disease Control (CDC) among recipients of blood that was pre-tested and found negative for antibody to the HIV-1 virus.

15 Clerical errors and other mistakes further expose patients to contaminated, incorrectly tested or mislabeled blood. To complicate the problem, one bad unit can create several victims, since whole blood is routinely split into components. Mistakes are not infrequent in blood banks. Since the beginning of 1990, 29,586 blood bank errors and accidents have been reported to the FDA. "How Safe Is 20 Our Blood," U.S. News and World Report, June 27, 1994, 68-78. Recalls by blood centers of blood released in error are generally ineffective because they take place months or years after the blood products have been transfused.

An alternative approach to eliminate transmission of diseases through 25 blood products is to develop a means to inactivate pathogens in transfusion products. Some of these techniques such as heat [J. Hilfenzous *et al.* J. Biol. Std. 70:589 (1987)], solvent/detergent treatment [B. Horowitz *et al.* Transfusion 25:516 (1985)], gama-irradiation [G. Moroff *et al.* Transfusion 26:453 (1986)] or UV alone [K.N. Proudouz *et al.* Blood 70:589 (1987)] are completely incompatible with maintenance of red cell function.

30 Another means to inactivate pathogens is the use of methylene blue. S.J. Wagner *et al.* examined methylene blue as a virucidal for red cell solutions. S.J. Wagner *et al.* Transfusion 33:30 (1993). Photo treatment of red cells with methylene blue was found to cause loss of ATP, enhanced ion permeability, and binding of autologous immunoglobulin (IgG) to the red cell surface. It was 35 speculated that some general (and undesirable) modification of the red cell membrane occurs as a result of the treatment.

Yet another approach is to deplete the red cell product of contaminating lymphocytes which may harbor viral pathogens. Both leukodepletion with filters and freeze/thaw procedures have been examined. S.M. Bruisten *et al.* Transfusion 30:833 (1990). Complete removal of lymphocytes, however, cannot 5 be achieved with such methods. Furthermore, leukodepletion does not address cell-free virus. Thus, this approach is not sufficient to render blood completely safe.

Finally, there is the approach of avoiding blood and using blood substitutes. Hemoglobin solutions, perfluorocarbon emulsions and vesicle-10 encapsulated hemoglobin have all been suggested as candidates. Unfortunately, each of these has been shown to be inadequate as a general substitute. See R.M. Winslow In: Blood Safety: Current Challenges (S.J. Nance ed.) (AABB 1992) (pp. 151-167).

In sum, there is a need for a means of inactivating viral pathogens in red 15 blood cell solutions. This approach must be effective without causing harm to the blood product or the transfusion recipient.

SUMMARY OF THE INVENTION

The present invention generally relates to new compounds and methods 20 for the *in vitro* inactivation of pathogens in biological material intended for *in vitro* or *in vivo* use, and in particular the inactivation of pathogens in solutions containing red blood cells, prior to clinical testing or transfusion.

In accordance with the present invention, a compound having a nucleic acid 25 binding ligand and a mustard group is selectively employed to treat contamination by nucleic acid-containing microorganisms, including pathogenic viruses. Without intending to suggest a mechanism for the present invention, such compounds are alkylating agents.

The present invention contemplates a method of inactivating pathogens in a blood product, comprising: adding a compound having a mustard group 30 and a nucleic acid binding ligand, selected from the group comprising a psoralen group and an acridine group, to a blood product suspected of containing pathogens, to create a mixture, said compound reaching a final concentration sufficient to inactivate substantially all of said pathogens, and incubating said mixture without significant damage to said blood product, to create an incubated 35 mixture. The present invention further contemplates the following specific embodiments of the invention: where the compound is added to the blood

product to a final concentration of the compound of between 1 μ g/ml and 250 μ g/ml.

The present invention further contemplates the following independent embodiments: where the compound is added to the blood product to a final concentration of the compound of between 1 μ g/ml and 250 μ g/ml; where the mixture is incubated for between 1 minute and 48 hours; where the mixture is incubated for between approximately 12 and 24 hours; where when the compound is added to the blood product, the compound is in a mixture comprising dextrose, sodium chloride, mannitol, adenine and H₂O; where the method further comprising: transfusing the incubated mixture into a mammal; where the blood product comprises red blood cells; where the method further comprises washing the blood product to remove compound from the incubated mixture; where the pathogens comprise viral pathogens or bacterial pathogens; where the compound is selected from the group consisting of: 8-[3-(Bis-2-chloroethyl) amino]propyloxysoralen, 5-[3-(Bis-2-chloroethyl) aminopropoxy]methyl-8-methoxysoralen, 5-[3-(Bis-2-chloroethyl) aminopropoxy]methyl-8-methoxysoralen, 4'-[4-(Bis-2-chloroethyl)aminobutoxy]methyl-4,5',8-trimethylpsoralen, and N1,N1-bis (2-chloroethyl)-N4-(6-chloro-2-methoxy-9-acridinyl)-1,4-pentanediamine; where more than one of the compounds is added to the blood product; where the method further comprises: removing the compound from the incubated mixture with an adsorbent material.

An alternative embodiment of the present invention comprises a method of inactivating pathogens in a blood product, comprising: adding a compound having a mustard group and a nucleic acid binding ligand, selected from the group consisting of a psoralen group and an acridine group, to a blood product comprising red blood cells suspected of containing pathogens, to create a mixture, the compound reaching a final concentration sufficient to inactivate substantially all of the pathogens, and incubating the mixture for between 1 minute and 48 hours, without significant damage to the red blood cells, to create an incubated mixture.

The present invention further contemplates the following independent embodiments of this alternative embodiment: where the compound is added to the blood product to a final concentration of the compound of between 1 μ g/ml and 250 μ g/ml; where when the compound is added to the blood product, the compound is in a solution comprising dextrose, sodium chloride, mannitol,

adenine and H₂O; where the method further comprises transfusing the incubated mixture into a mammal; where the pathogens comprise viral pathogens; where the pathogens comprise bacterial pathogens; where the method further comprises washing the blood product to remove compound

5 from the incubated mixture; where the compound is selected from the group consisting of: 8-[3-(Bis-2-chloroethyl) amino]propyloxysoralen, 5-[3-(Bis-2-chloroethyl) aminopropoxy]methyl-8-methoxysoralen, 5-[3-(Bis-2-chloroethyl) aminopropoxy]methyl-8-methoxysoralen, 4'-[4-(Bis-2-chloroethyl)aminobutoxy]methyl-4,5',8-trimethylpsoralen, and N1,N1-bis (2-

10 chloroethyl)-N4-(6-chloro-2-methoxy-9-acridinyl)-1,4-pentanediamine; where more than one of the compounds is added to the blood product; where the method further comprises removing the compound from the incubated mixture with an adsorbent material.

The present invention contemplates an improved method of treating biological compositions, comprising: adding a compound having a nucleic acid binding ligand and a mustard group to a biological composition to create a mixture, and incubating the mixture to create an incubated mixture.

The present invention further contemplates the following independent embodiments of the improved method: where the compound is added to the biological composition to a final concentration of the compound of between 1 μ g/ml and 250 μ g/ml; where the mixture is incubated for between 1 minute and 48 hours; where when the compound is added to the biological composition, the compound is in a mixture comprising dextrose, sodium chloride, mannitol, adenine and H₂O; where the biological composition comprises a blood product; where the method further comprises transfusing the incubated mixture into a mammal; where the blood product comprises red blood cell; where the method further comprises, washing the blood product to remove the compound from the incubated mixture; where the blood product further comprises viral pathogens; where the blood product further comprises bacterial pathogens; where the compound is selected from the group consisting of: 8-[3-(Bis-2-chloroethyl) amino]propyloxysoralen, 5-[3-(Bis-2-chloroethyl) aminopropoxy]methyl-8-methoxysoralen, 5-[3-(Bis-2-chloroethyl) aminopropoxy]methyl-8-methoxysoralen, 4'-[4-(Bis-2-chloroethyl)aminobutoxy]methyl-4,5',8-trimethylpsoralen, and N1,N1-bis (2-

20 chloroethyl)-N4-(6-chloro-2-methoxy-9-acridinyl)-1,4-pentanediamine; where the compound is selected from the group consisting of: 8-[3-(Bis-2-chloroethyl) amino]propyloxysoralen, 5-[3-(Bis-2-chloroethyl) aminopropoxy]methyl-8-methoxysoralen, 5-[3-(Bis-2-chloroethyl) aminopropoxy]methyl-8-methoxysoralen, 4'-[4-(Bis-2-chloroethyl)aminobutoxy]methyl-4,5',8-trimethylpsoralen, and N1,N1-bis (2-

25 chloroethyl)-N4-(6-chloro-2-methoxy-9-acridinyl)-1,4-pentanediamine; where the compound is selected from the group consisting of: 8-[3-(Bis-2-chloroethyl) amino]propyloxysoralen, 5-[3-(Bis-2-chloroethyl) aminopropoxy]methyl-8-methoxysoralen, 5-[3-(Bis-2-chloroethyl) aminopropoxy]methyl-8-methoxysoralen, 4'-[4-(Bis-2-chloroethyl)aminobutoxy]methyl-4,5',8-trimethylpsoralen, and N1,N1-bis (2-

30 chloroethyl)-N4-(6-chloro-2-methoxy-9-acridinyl)-1,4-pentanediamine; where the compound is selected from the group consisting of: 8-[3-(Bis-2-chloroethyl) amino]propyloxysoralen, 5-[3-(Bis-2-chloroethyl) aminopropoxy]methyl-8-methoxysoralen, 5-[3-(Bis-2-chloroethyl) aminopropoxy]methyl-8-methoxysoralen, 4'-[4-(Bis-2-chloroethyl)aminobutoxy]methyl-4,5',8-trimethylpsoralen, and N1,N1-bis (2-

35 chloroethyl)-N4-(6-chloro-2-methoxy-9-acridinyl)-1,4-pentanediamine; where the compound is selected from the group consisting of: 8-[3-(Bis-2-chloroethyl) amino]propyloxysoralen, 5-[3-(Bis-2-chloroethyl) aminopropoxy]methyl-8-methoxysoralen, 5-[3-(Bis-2-chloroethyl) aminopropoxy]methyl-8-methoxysoralen, 4'-[4-(Bis-2-chloroethyl)aminobutoxy]methyl-4,5',8-trimethylpsoralen, and N1,N1-bis (2-

method further comprises removing the compound from the incubated mixture with an adsorbent material.

The present invention contemplates additionally an improved method of treating clinical samples, comprising, in the following order: providing a compound having a mustard group and a nucleic acid binding ligand, selected from the group consisting of a psoralen group and an acridine group, and a clinical sample intended for *in vitro* clinical testing; adding the compound to the clinical sample, to create a mixture, incubating the mixture for between 1 minute and 48 hours, and measuring the level of a clinical chemistry analyte in the clinical sample.

The present invention further contemplates the following independent embodiments of the improved method: where the compound is selected from the group consisting of: 8-[3-(Bis-2-chloroethyl) amino]propyloxypsoralen, 5-[3-(Bis-2-chloroethyl) aminopropoxy]methyl-8-methoxypsoralen, 5-[3-(Bis-2-chloroethyl) aminopropoxy]methyl-8-methoxypsoralen, 4'-[4-(Bis-2-chloroethyl)aminobutoxy]methyl-4,5',8-trimethylpsoralen, and N1,N1-bis (2-chloroethyl)-N4-(6-chloro-2-methoxy-9-acridinyl)-1,4-pentanediamine; where the clinical sample comprises red blood cells; where the red blood cells further comprise viral pathogens or bacterial pathogens; where the incubation step is performed without significant damage to the clinical chemistry analog.

The present invention contemplates a method of inactivating pathogens in a red blood cell containing composition, comprising: adding a compound having a nucleic acid binding ligand and a mustard group to a blood product comprising red blood cells, where the blood product is suspected of containing pathogens, to create a mixture, the compound reaching a final concentration sufficient to inactivate substantially all of the pathogens, incubating the mixture *in vitro* for between 1 minute and 48 hours while retaining the ability of the blood product to function for the purpose the blood product was prepared, to create an incubated mixture, and transfusing the incubated mixture into a mammal.

The present invention further contemplates the following independent embodiments of the improved method: where the compound is added to the blood product to a final concentration of the compound of between 1 μ g/ml and 250 μ g/ml; where when the compound having a nucleic acid binding ligand and a mustard group is added to the blood product, the compound is in a solution comprising dextrose, sodium chloride, mannitol, adenine and H₂O; where the

pathogens comprise viral pathogens; where the pathogens comprise bacterial pathogens; where the compound is selected from the group consisting of: 8-[3-(Bis-2-chloroethyl) amino]propyloxypsoralen, 5-[3-(Bis-2-chloroethyl) aminopropyloxy]methyl-8-methoxypsoralen, 5-[3-(Bis-2-chloroethyl) aminopropyloxy]methyl-8-methoxypsoralen, 4'-[4-(Bis-2-chloroethyl)aminobutoxy]methyl-4,5',8-trimethylpsoralen, and N1,N1-bis (2-chloroethyl)-N4-(6-chloro-2-methoxy-9-acridinyl)-1,4-pentanediamine; where more than one of the compounds is added to the blood product; where the method further comprises removing the compound from the incubated mixture with an adsorbent material; where the nucleic acid binding ligand of the compound is selected from the group comprising: a psoralen group and a 9-aminoacridine group

The present invention contemplates a specific embodiment of the present invention, a method of inactivating pathogens in a red blood cell containing composition, comprising adding a compound having an acridine group and a mustard group to a blood product comprising red blood cells, where the blood product is suspected of containing pathogens, to create a mixture, the compound reaching a final concentration sufficient to inactivate substantially all of the pathogens, and incubating the mixture in vitro for between 1 minute and 48 hours, while retaining the ability of the blood product to function for the purpose the blood product was prepared, to create an incubated mixture

The present invention further contemplates the following independent embodiments of the improved method: where the compound is added to the blood product comprising red blood cells to a final concentration of between 1 μ g/ml and 250 μ g/ml; where when the compound is added to the blood product comprising red blood cells, the mixture further comprises dextrose, sodium chloride, mannitol, adenine and H₂O; where the method further comprises transfusing the mixture into a mammal; where the pathogens comprise viral pathogens; where the pathogens comprise bacterial pathogens; where the compound is N1,N1-bis (2-chloroethyl)-N4-(6-chloro-2-methoxy-9-acridinyl)-1,4-pentanediamine; where the method further comprises removing the compound from the incubated mixture with an adsorbent material.

The present invention further comprises the following novel compositions of matter: 8-[3-(Bis-2-chloroethyl) amino]propyloxypsoralen; 5-[3-(Bis-2-chloroethyl) aminopropyloxy]methyl-8-methoxypsoralen; 5-[3-(Bis-2-chloroethyl) aminopropyloxy]methyl-8-methoxypsoralen; 4'-[4-(Bis-2-

chloroethyl)aminobutoxy]methyl-4,5',8-trimethylpsoralen; and, 5-[N,N-bis(2-chloroethyl)amino]methyl-8-methoxypsoralen. It is contemplated that in various embodiments of the present invention the treated blood product or biological sample may be mixed with the compound prior to or during 5 incubation.

DESCRIPTION OF THE FIGURES

FIG. 1 is a graph showing reduction in titer of R17 treated with varying 10 concentrations of quinacrine mustard in either Adsol or dimethyl sulphoxide (DMSO). The horizontal dotted line represents the limit of detection of the assay used.

FIG. 2 is a graph showing inactivation of R17 by quinacrine mustard.

FIG. 3 is a graph showing the inactivation kinetics of quinacrine mustard.

FIG. 4 is a graph showing the reduction in titer of R17 as a function of time 15 of incubation of quinacrine mustard in Adsol.

FIG. 5 is a graph showing the reduction in R17 inactivation activity as a function of time when incubated in the presence of either Adsol, red blood cells or Amberlite XAD-16™.

FIG. 6 is a graph showing the effects of quinacrine mustard at varying 20 concentrations on extra-cellular potassium levels.

FIG. 7 is a graph showing the reduction in titer of R17 in packed red blood cells treated with varying concentrations of quinacrine mustard; the horizontal dotted line represents the limit of detection of the assay used.

FIG. 8 is a graph showing the activity of quinacrine mustard, after 25 incubation in red blood cells, with or without the presence of Amberlite XAD-16™, in an Ames assay using strain TA 1537.

FIG. 9 is a graph showing the inactivation of a bacterial strain, *Staphylococcus Epidermidis*, using quinacrine mustard at varying concentrations.

30 DESCRIPTION OF THE INVENTION

The present invention generally relates to new compounds and methods for the *in vitro* inactivation of pathogens in biological material intended for *in vitro* or *in vivo* use, and in particular the inactivation of pathogens in solutions containing red blood cells, prior to clinical testing or transfusion. In accordance 35 with the present invention, a compound having a nucleic acid binding ligand and a mustard group is selectively employed to treat contamination by nucleic acid-containing microorganisms, including pathogenic viruses and bacteria.

I. COMPOUNDS OF THE PRESENT INVENTION

Red blood cell decontamination methods using photoactivated compounds have in the past encountered a problem due to the absorbency, by hemoglobin, of light at wavelengths necessary to activate compounds. Even 5 compounds with a primary absorbance peak greater than 600 nm (the long-wavelength limit of hemoglobin) have decreased efficacy in red blood cell concentrates due to scattering of light by the cells. Thus, even though the previous methods would inactivate pathogens in other media, they are inefficient in the presence of red blood cells. In contrast, the present invention 10 contemplates a method of sterilization capable of effectively inactivating pathogens even in red cell concentrates [hematocrits ranging from 1% to 60% or higher].

The present invention contemplates treating red blood cell solutions with a compound which inactivates pathogens without requiring exposure to light. 15 The advantage of the present invention for inactivation in fluids for transfusion is two-fold. First, light is not required, allowing for a less complex technology for inactivation. Second, the decontamination compound has reacted after a short amount of time. The treatment is complete in several minutes or hours, depending on the compound used. In one embodiment, material which does 20 not react with nucleic acids or another biomolecule hydrolyzes, leaving little or no residual compound to be transfused. In another embodiment, residual compound or chemical product of the reaction is removed by adsorption or washing, as described below.

Without intending to be limited to any particular mechanism of action of 25 the present invention, compounds of the present invention have two characteristics in common. The first characteristic is that they bind nucleic acid non-covalently. The second is that they have at least one mustard group.

A. Non-covalent Nucleic Acid Binding Group

A compound which binds nucleic acid has a "nucleic acid binding ligand", 30 herein defined as a group which has an affinity for and can bind to nucleic acids non-covalently. There are several modes of binding to nucleic acids. Compounds which bind by any of the following modes, combinations of them, or other modes are considered nucleic acid binding ligands. While the 35 invention is not limited to the following compounds, some examples of nucleic acid binding ligands are: a) intercalators, such as acridines, acridones, proflavin,

acriflavine, actinomycins, anthracyclinones, beta-rhodomycin A, daunamycin, thiaxanthenones, miracil D, anthramycin, mitomycin, echinomycin, quinomycin, triostin, diacridines, ellipticene (including dimers, trimers and analogs), norphilin A, fluorenes and flourenones, fluorenodiamines, quinacrine, 5 benzacridines, phenazines, phenanthradines, phenothiazines, chlorpromazine, phenoxazines, benzothiazoles, xanthenes and thio-xanthenes, anthraquinones, anthrapyrazoles, benzothiopyranoindoles, 3,4-benzpyrene, benzopyrene diol epoxidie, 1-pyrenyloxirane, benzanthracene-5,6-oxide, benzodipyrrones, benzothiazoles, quinolones, chloroquine, quinine, phenylquinoline 10 carboxamides, furocoumarins, such as psoralens and isopsoralens, ethidium salts, propidium, coralyne, ellipticine cation and derivatives, polycyclic hydrocarbons and their oxirane derivatives, and echinimycin; b) minor groove binders such as distamycin, mitomycin, netropsin, other lexitropsins, Hoechst 33258 and other Hoechst dyes, DAPI (4',6'-diamidine-2-phenylindole), berenil, 15 and triarylmethane dyes; c) major groove binders such as aflatoxins; d) molecules that bind by electrostatics (phosphate backbone binders), such as spermine, spermidine, and other polyamines; e) nucleic acids or analogues which bind by such sequence specific interactions as triple helix formation, D-loop formation, and direct base pairing to single stranded targets.

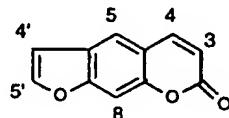
20 While not limited to any particular mechanism, it is believed that the nucleic acid binding ligand functions as a carrier (or anchor) that targets (or directs) the molecule to nucleic acid, interacting non-covalently therewith.

1. Psoralens as Noncovalent Nucleic Acid Binding Groups

25 The present invention contemplates a specific class of compounds which use a psoralen group as a nucleic acid binding group. These compounds are particularly suitable for use in the present invention. Previous nucleic acid specific alkylating agents typically contain an alkylating moiety such as a chloroethylamine fragment, connected to a nucleic acid specific group, an 30 intercalator (e.g., acridine), or a minor groove binder. These moieties are mutagenic in themselves. After the residual alkylating agent has been hydrolysed from the compound, the residue may still be rather mutagenic. In contrast, compounds having a psoralen nucleic acid binding group display substantially reduced mutagenicity, thus providing an improved safety factor. Psoralens are 35 well known as nucleic acid intercalators but their utility has mainly been as photoactive agents which covalently bind to the nucleic acids upon irradiation

with UVA (320-400nm). Without intending to be bound to any mechanism of action of the present invention, it is hypothesized that the role of the psoralen group in the compounds described here is as an intercalator to increase the specificity of location of the alkylating agent, and thus the specificity of the alkylation reaction to nucleic acids.

5 Specifically some novel compounds of the present invention contain psoralens [7H-furo(3,2-g)-(1)-benzopyran-7-one, or β -lactone of 6-hydroxy-5-benzofuranacrylic acid], which are linear molecules:



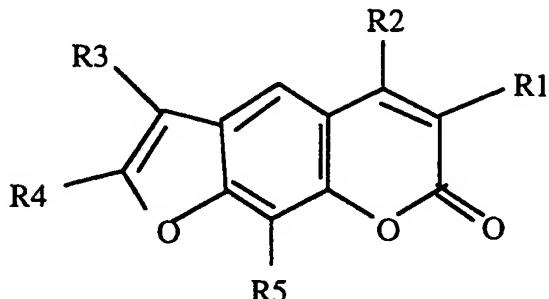
10 and in which the two oxygen residues appended to the central aromatic moiety have a 1, 3 orientation, and further in which the furan ring moiety is linked to the 6 position of the two ring coumarin system. Psoralen derivatives are derived from substitution of the linear furocoumarin at the 3, 4, 5, 8, 4', or 5' positions.

15 A psoralen of the present invention is represented in the structure below, wherein one or two of R₁, R₂, R₃, R₄, or R₅ are a (2-chloroethyl)amino group, optionally with a second 2-chloroethyl group on the amine, attached to a psoralen by a chain of one to nine carbons. The chain can contain one or more heteroatoms of the group comprising oxygen, nitrogen or sulfur. The chain can optionally contain one or more unsaturated bonds or carbonyl groups. The 20 chain is optionally substituted with lower alkyl groups.

25 Specifically the novel compounds contain a psoralen where one or two of R₁, R₂, R₃, R₄, or R₅ are a (2-chloroethyl)amino group or (2-bromoethyl)amino group, optionally with a second 2-haloethyl group on the amine, attached to a psoralen by a chain of one to nine carbons. The chain can contain one or more heteroatoms of the group comprising oxygen, nitrogen or sulfur. The chain can optionally contain one or more unsaturated bonds or carbonyl groups. The chain is optionally substituted with lower alkyl groups.

30 Positions R₁ - R₅ unoccupied by the alkylating group may be hydrogen, lower alkyl, lower alkoxy, halogen, CH₂OR₆ or CH₂NR₇R₈ (where R₆-R₈ are hydrogen or lower alkyl).

The compounds may be neutral amines, or their salts.



Ring construction of psoralens, and their functionalization with displaceable groups, X, (where X = Cl, Br, I, OSO₂CH₃, etc,) is described in the literature (Hearst et al, US patents 4,124,598; 4,196,281; Kaufman, US patents 4,269,851; 4,269,852; 4,294,822; 4,298,614; 4,370,344; Wollowitz et al., US patent 5,399,719; Antonello, S. C., et al., *Farmaco* (1978) 34, 139).

The desired products are constructed by one of three routes. In the first, a 2-hydroxyethylamine (e.g., diethanolamine) is reacted directly with psoralen-(CH₂)_nX where X is a readily displaceable group such as a halide, mesylate or tosylate. The chain is attached to the psoralen at the 3,4,4',5', or 8 positions, other substituents may be on the psoralen ring, and n = 1-6. In a second steps, the hydroxy groups of the intermediate are then converted to chloro or bromo groups by standard means, for example with thionyl chloride to give the desired product.

In the second route, the functionalized psoralen is reacted with HY-(CH₂)_m-OH, where Y = NH, S, O and m = 2-6. The terminal alcohol is then converted to a readily displaceable group (halo, mesylate, etc.) by standard means, then reacted with the (2-hydroxyethyl)amine. The resultant compound is converted into a haloethylamine-functionalized product as described above.

In the final route, the HY-(CH₂)_m-N(CH₂CH₂OH)₂, where m= 2 to 6, is prepared as described in the literature (e.g., Peck, R. M., Preston, R. K., Creech, H. J., *J. Amer. Chem. Soc.*, (1959) 81, 3984), and reacted directly with the functionalized psoralen. Again, conversion of the hydroxy groups to halides gives the desired psoralen mustard products.

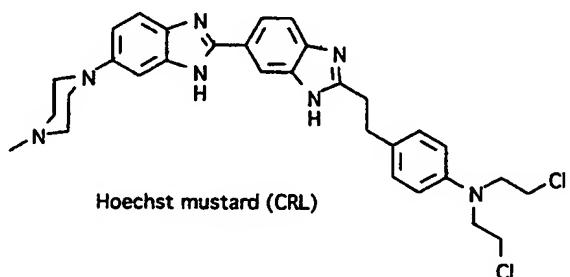
B. Mustard Group

The second characteristic that compounds of the present invention have in common is that they contain at least one mustard group. A "mustard group"

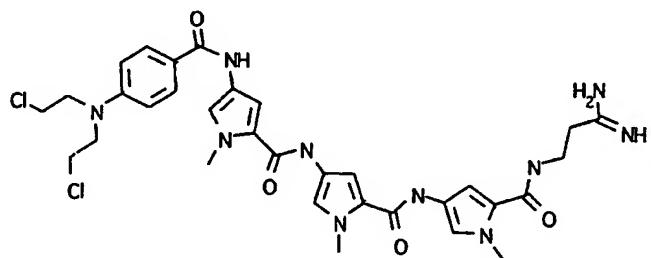
is defined here as including mono or bis haloethylamine groups, and mono haloethylsulfide groups.

The present invention is not limited strictly to mustards. It is believed that mustards can form reactive intermediates such as aziridinium or aziridine complexes and sulfur analogs of these complexes. The present invention also 5 contemplates functional groups that are the equivalent of mustards, such as epoxides.

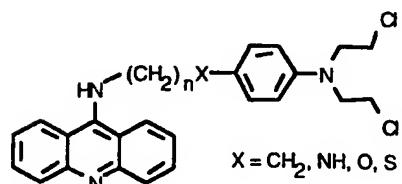
While not limited to any particular mechanism, compounds having mustard groups are known to react with nucleic acids to form covalent 10 complexes which inhibit nucleic acid replication. They are typically solids that, upon dissolution in a medium which contains nucleophiles, completely react within minutes or hours. Some examples are shown below (where n is a whole number from 1-10).

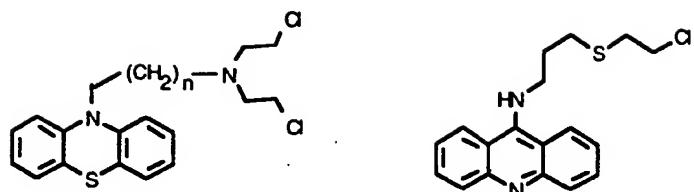


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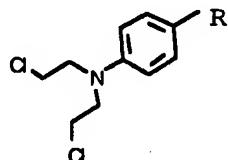
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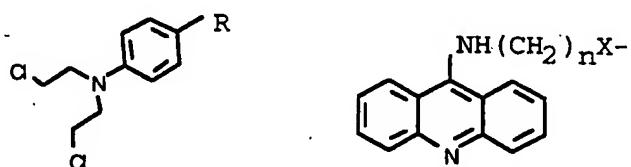


Nitrogen mustards are thoroughly described in the literature. E.g., see Gravatt, G.L., et al., "DNA-Directed Alkylating Agents. 4. 4-Anilinoquinoline-Based Minor Groove Directed Aniline Mustards," J. Med. Chem. 34:1552 (1991); Cummings, J., et al., "Determination of Reactive Nitrogen Mustard Anticancer Drugs in Plasma by High-Performance Liquid Chromatography Using Derivatization," Anal. Chem. 63:1514 (1991). They are known to be potent alkylators of nucleic acid and due to this mode of action, they have been widely studied as anti-tumor agents. Several have found practical use in the clinic (e.g. aniline mustard, chlorambucil, melphalan).

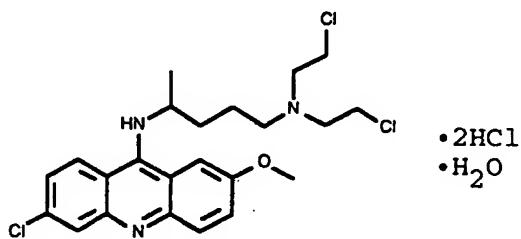
One class of nitrogen mustards is the aniline mustard class. These compounds have at least one haloethylaminoaniline group, where the haloethyl may be mono or bis. An example of a bis(haloethyl)aminoaniline group appears 15 below (where R is the point of linkage to other groups):



A specific aniline mustard group is the acridine carried aniline mustards (described in Gravatt, et al., J. Med. Chem. 34:1552), where R comprises a linking 20 group (for example O, CH₂, S, COHN, or CO, however, other linking groups are contemplated) which links the mustard group to a second component, an acridine group. An example of the components of a 9-aminoacridine carried aniline mustard appears below (where X is the linking group):



The present invention demonstrates that a specific compound having a nucleic acid binding ligand and a mustard group, N1,N1-bis (2-chloroethyl)-N4-(6-chloro-2-methoxy-9-acridinyl)-1,4-pentanediamine dihydrochloride ("quinacrine mustard"), is useful as an antiviral agent for red cells. Quinacrine mustard is commercially available (from Aldrich, Milwaukee, WI, as quinacrine mustard dihydrochloride hydrate, structure shown below).



10

II. MATERIALS FOR DECONTAMINATION

The present invention contemplates novel compounds and a new use for compounds having a nucleic acid binding ligand and a mustard group: the inactivation of viruses and bacteria in blood, blood products and other biological compositions. While not an exclusive list, the following biological compositions are contemplated, and are referred to generally as "samples". Of the blood and blood components contemplated, exemplary compositions include whole blood, packed red cells, platelets, plasma (fresh or fresh frozen plasma), and proteins derived from blood or blood components. Blood components also encompass plasma protein portion, antihemophilic factor (AHF, Factor VIII); Factor IX and Factor IX complex (Factors II, VII, IX and X); fibrinogens, Factor XIII, prothrombin and thrombin (Factor II and IIa); immunoglobulins (e.g. IgA, IgD, IgE, IgG and IgM and fragments thereof e.g. Fab, F(ab')₂, and Fc); hyper-immune globulins as used against tetanus and hepatitis B; cryoprecipitate; albumin; interferons; lymphokines; and transfer factors. The present invention also contemplates, as part of blood and blood products, a synthetic version of any blood or blood product.

Other biological compositions which are contemplated by the present invention include vaccines, recombinant DNA produced proteins, oligopeptide ligands, etc. Biological compositions also encompass clinical samples other than

blood and blood components, such as urine, sputum, feces, spinal fluid, and other materials removed from mammals for clinical testing.

5

III. INACTIVATION OF PATHOGENS

10 The present invention contemplates treating a blood product with a compound having a nucleic acid binding ligand and a mustard group to inactivate contaminating pathogen nucleic acid sequences before using the blood product.

A. Inactivation In General

15 The term "inactivation" is here defined as the altering of the nucleic acid of a unit of pathogen so as to render the unit of pathogen incapable of replication. This is distinct from "total inactivation", where all pathogen units present in a given sample are rendered incapable of replication, or "substantial inactivation," where most of the pathogen units present are rendered incapable of replication. "Inactivation efficiency" of a compound is defined as the level of 20 inactivation the compound can achieve at a given concentration of compound. For example, if 100 μ M of a hypothetical compound X inactivated 5 logs of HIV virus whereas under the same experimental conditions, the same concentration of compound Y inactivated only 1 log of virus, then compound X would have a better "inactivation efficiency" than compound Y.

25 To appreciate that an "inactivation" method may or may not achieve "total inactivation," it is useful to consider a specific example. A bacterial culture is to be inactivated if an aliquot of the culture, when transferred to a fresh culture plate and permitted to grow, is undetectable after a certain time period. A minimal number of viable bacteria must be applied to the plate for a signal to be 30 detectable. With the optimum detection method, this minimal number is 1 bacterial cell. With a sub optimal detection method, the minimal number of bacterial cells applied so that a signal is observed may be much greater than 1. The detection method determines a "threshold" below which the "inactivation method" appears to be completely effective (and above which "inactivation" is, 35 in fact, only partially effective).

B. Inactivation of Potential Pathogens

The same considerations of detection method and threshold exist when determining the sensitivity limit of an inactivation method for nucleic acid. Again, "inactivation" means that a unit of pathogen is rendered incapable of

5 replication.

In the case of inactivation methods for material to be used by humans, whether *in vivo* or *in vitro*, the detection method can theoretically be taken to be the measurement of the level of infection with a disease as a result of exposure to the material. The threshold below which the inactivation method is complete

10 is then taken to be the level of inactivation which is sufficient to prevent disease from occurring due to contact with the material. It is recognized that in this

practical scenario, it is not essential that the methods of the present invention result in "total inactivation". That is to say, "substantial inactivation" will be adequate. Thus "substantially all" of a pathogen is inactivated when any viable

15 portion of the pathogen which remains is insufficient to cause disease. The inactivation method of the present invention renders nucleic acid in pathogens substantially inactivated. In one embodiment, the inactivation method renders pathogen nucleic acid in blood preparations substantially inactivated.

Without intending to be limited to any method by which the compounds

20 of the present invention inactivate pathogens, it is believed that inactivation results from alkylation of portions of the pathogen nucleic acid. Further, while it is not intended that the inactivation method of the present invention be limited by the nature of the nucleic acid; it is contemplated that the inactivation method

25 render all forms of nucleic acid (whether DNA, mRNA, etc.) substantially inactivated.

When a compound having a nucleic acid binding ligand and a mustard group is used to modify nucleic acid, the interaction of the pathogen nucleic acid (whether DNA, mRNA, etc.) with the compound preferably prevents replication of the pathogen, such that, if a human is exposed to the treated pathogen,

30 infection will not result.

"Synthetic media" is herein defined as an aqueous synthetic blood or blood product storage or processing media. In one embodiment, the present invention contemplates inactivating blood products in synthetic media comprising a buffered saline solution.

35 The present method inactivates nucleic acid based pathogens present in blood through a single procedure. Thus, it has the potential to eliminate

bacteria, protozoa, and viruses as well. It is not intended that the present invention be limited by the number or nature of pathogens inactivated. Importantly, however, the treatment of the present invention has been found to block the replication of the HIV virus. Had an effective decontamination 5 method been available prior to the advent of the AIDS pandemic, no transfusion associated HIV transmission would have occurred. Decontamination based on compounds having a nucleic acid binding ligand and a mustard group has the potential to eliminate all infectious agents from the blood supply, regardless of the pathogen involved.

10

C. Selecting Compounds for Inactivation of Pathogens

In order to evaluate a compound to decide if it would be useful in the decontamination methods of the present invention, two important properties should be considered: 1) the compound's ability to inactivate pathogens, 2) its 15 mutagenicity after treatment, and 3) the ability of the treatment to perform steps 1) and 2) while retaining the ability of said blood product to function for the purpose said blood product was prepared. The ability of a compound to inactivate pathogens may be determined by several methods. One technique is to perform a bacteriophage screen, an assay which determines nucleic acid 20 binding of test compounds. A screen of this type, an R17 screen, is described in detail in an example, below. If the R17 screen shows inactivation activity, it is useful to directly test the compound's ability to inactivate a virus. One method of performing a direct viral inactivation screen is described in detail in an example below for cell free HIV.

25

The R17 bacteriophage screen is believed to be predictive of HIV inactivation efficiency, as well as the efficiency of compounds against many other viruses. R17 was chosen because it was expected to be a very difficult pathogen to inactivate. It is a small, single stranded RNA phage. Without intending to be limited to any means by which the present invention operates, it is expected that 30 shorter pieces of nucleic acid are harder to inactivate because they provide a smaller target for the compound. Thus it is expected that under conditions that result in the inactivation of R17 the inactivation of many viruses and bacteria will also be obtained.

35

The cell free HIV screen complements the R17 screen by affirming that a given compound which has tested positive in R17 will actually work effectively

to inactivate viruses. Thus, if a compound shows activity in the R17 screen, it is next tested in the viral inactivation screen.

The second property that is important in testing a compound for use in methods of the present invention is mutagenicity after treatment. The most 5 widely used mutagen/carcinogen screening assay is the Ames test. This assay is described by D.M. Maron and B.N. Ames in *Mutation Research*, 113: 173 (1983) and a specific screen is described in detail in an example, below. The Ames test utilizes several unique strains of *Salmonella typhimurium* that are histidine-dependent for growth and that lack the usual DNA repair enzymes. The 10 frequency of normal mutations that render the bacteria independent of histidine (i.e., the frequency of spontaneous revertants) is low. The test allows one to evaluate the impact of any residual chemical entities that remain after treatment on this revertant frequency.

Because some substances are not mutagenic by themselves, but are 15 converted to a mutagen by metabolic action, the compound to be tested is mixed with the bacteria on agar plates along with the liver extract. The liver extract serves to mimic metabolic action in an animal. Control plates have only the bacteria and the extract.

The mixtures are allowed to incubate. Growth of bacteria (if any) is 20 checked by counting colonies. A positive Ames test is one where the number of colonies on the plates with mixtures containing the compound significantly exceeds the number on the corresponding control plates.

When known carcinogens are screened in this manner with the Ames test, approximately ninety percent are positive. When known noncarcinogens 25 are similarly tested, approximately ninety percent are negative.

A compound (X) can be evaluated as a potential decontamination compound for use in the present invention, as shown in Table 1, below. X is initially evaluated in Step I. X is screened in the R17 assay, in the presence of red blood cells, at several different concentrations between 4 and 320 μ M, as 30 explained in an example below. If the compound shows inactivation activity greater than 1 log inactivation of R17 (log kill) in the R17 screen at any concentration, the compound is then screened in the cell free HIV assay, Step II, as explained in an example below. If the compound shows inactivation activity greater than 1 log inactivation of HIV (log kill) in the cell free HIV assay, the 35 compound is a useful agent for inactivation of pathogens in clinical test samples. If the compound is being evaluated for decontamination of biological materials

to be used *in vivo*, it is then taken through Step III. A biological material decontaminated by a method of the present invention is screened in the Ames assay to determine whether any compound that remains after decontamination is mutagenic. Finally, if the residual material does not show significant 5 mutagenicity in the Ames assay, the compound is identified as a useful agent for inactivation of pathogens in products to be used *in vivo* as well.

TABLE 1

STEP	SCREEN	RESULT	INTERPRETATION
I	R17	>1 log kill by any concentration	potential compound, go to step 2
		<1 log kill	compound is ineffective as an inactivation treatment
II	Viral Inactivation	> 1 log kill by any concentration	useful for clinical sample decontamination go to step 3
		< 1 log kill	compound is ineffective as an inactivation treatment
III	Ames	less mutagenic than AMT	useful agent for inactivation

10 The third property, retaining the ability of said blood product to function for the purpose said blood product was prepared, can be tested as follows. The treated blood product can be screened in one or more tests for blood product function that are generally acceptable. If a test sample is treated, screened and compared to a control sample, a determination can easily be made whether the 15 variation, if any from the control sample, are within an acceptable range according to current standards of blood banking practice. For example, to screen a particular compound for use in a method of the present invention to treat red blood cell concentrates, one can determine whether the treated red blood cells retain the ability to function for the purpose they are prepared by measuring the 20 stimulation of IgG binding in the agglutination experiment described in Example 10.

By following these instructions, a person can determine which compounds would be appropriate for use in methods of the present invention.

D. Delivery and Removal of Compounds for Inactivation

The present invention contemplates several different formulations and routes by which the compounds described herein can be delivered in an inactivation method, and where desired, removed. This section is merely illustrative, and not intended to limit the invention to any form or method of treatment with the compounds.

The compounds of the present invention may be introduced in an inactivation method in several forms and at various times, which may depend on the purpose for which the blood preparation is decontaminated. The 10 compounds may, for example, be introduced as an aqueous solution in water, saline, a synthetic media or a variety of other media. The compounds may alternatively be provided as dry formulations, with or without adjuvants. Further, the compounds may be introduced alone, or in a "cocktail" or mixture of several different compounds. In a preferred embodiment, a compound 15 having a nucleic acid binding ligand and a mustard group is employed at a concentration less than 250 μ M.

The compounds can be mixed directly with the blood or blood product or prepared as a solution or suspension in a bio-compatible fluid [such as Adsol (the 20 contents of which are set forth in the Experimental section, below) or an organic solvent (e.g. dimethyl sulfoxide (DMSO), ethanol, glycerin, polyethylene glycol (PEG) or polypropylene glycol)] and then mixed with the blood. The new compounds may also be provided at different points in the inactivation process. For example, the compound may be introduced to the reaction vessel, such as a blood bag, at the point of manufacture. Alternatively, the compound may be 25 added to the material to be sterilized after the material has been placed in the reaction vessel. For all methods of compound introduction, the compound is preferably mixed thoroughly with the sample to be decontaminated prior to or during incubation of the compound in the sample.

1. Decontamination of Clinical Samples.

30 A clinical sample is defined as any material removed from mammals for clinical testing, including, but not limited to blood and blood components, urine, sputum, feces, bone marrow, and spinal fluid. A serum analyte is defined here as a component found in clinical samples which is measured in clinical chemistry tests. Examples of serum analytes include, but are not limited to: 35 glucose, blood urea nitrogen, creatinine, blood urea nitrogen/creatinine ratio, sodium, potassium, chloride, magnesium, calcium, phosphorous inorganic, total

protein, albumin, total globulin, albumin/globulin ratio, bilirubin, alkaline phosphatase, lactate dehydrogenase, glutamate transferase, aspartate transaminase, alanine aminotransferase, uric acid, iron, triglycerides, and cholesterol.

5 In the decontamination of clinical samples, the goal is to decontaminate the sample so that infectious agents cannot be transferred to clinical laboratory workers. Because the samples will not be transfused into a recipient, there is less concern that residual compound be removed from the sample. Thus removal techniques may not be desired. The present invention contemplates that the 10 compound may be in the clinical sample test tube prior to drawing the sample from the patient, or it may be added after drawing. Once the compound has contacted the sample, the sample preferably is thoroughly mixed, then incubated. The sample may then be screened in the desired panel of clinical chemistry tests without concern for spreading infectious diseases.

15

2. Decontamination of Blood Products for Transfusion.

The compound for decontamination may be introduced to the whole blood prior to fractionating, by adding to the blood bag before or after blood is drawn. Alternatively, the compound may be added after fractionation of the 20 blood, decontaminating the individual fractions.

For the decontamination of particularly viscous blood products, such as red blood cell concentrates, active mixing of the blood product with the compound may be desirable to achieve rapid and complete distribution of the compound. The present invention contemplates embodiments in which a 25 compound of the present invention is actively mixed with the blood product to be treated prior to or during the incubation of the blood product with the compound. The following is a non-exclusive list of examples that the present invention contemplates as appropriate technologies for mixing: A flow system of mixing compounds with blood products is contemplated where standard 30 tubing fitting shapes such as T or Y shapes are used to provide orifices for enhanced mixing. These shapes may easily be optimized to enhance turbulence or create a swirling pattern at the point of mixing. One embodiment contemplates changing the direction of one or more flow streams for more energetic mixing. Concentric or annular flow streams are also contemplated. 35 A static mixer with pins and vanes is also contemplated to enhance mixing.

Thermally formed plastic tubing may be customized to enhance mixing, where potential configurations include, but are not limited to, alternating narrowings in the tubing to create turbulence and variations in placement of pinched segments to enhance mixing. The present invention also contemplates the use 5 of tubing that has been extruded into segments of larger and smaller diameter, such as bubble tubing. Helix shaped tubing is contemplated to provide secondary flow patterns. Enhanced mixing may also be achieved by causing the flow to pass through a mesh or screen. The mixing may also be achieved by a washing step, for which examples are provided below.

10 In products for transfusion, in some cases it may be desirable to remove residual compound or chemical products of the reaction after treatment of the product but prior to transfusion. The present invention contemplates the removal of the compound from the blood product prior to transfusion.

15 Adsorption Methods

In one embodiment, any residual compound or chemical product may be removed using an adsorbent material. Examples of adsorbent materials which may be used in the present invention include, but are not limited to: activated charcoal (either uncoated or coated with a polymer), silica, reverse phase silica, 20 polymeric adsorbents, and modified polymeric adsorbents. The present invention contemplates several ways for the introduction of the adsorbent material to the blood products for transfusion. The adsorbent may be mixed directly with the blood products and subsequently filtered out. Alternatively, the blood products could be passed through a filter containing the adsorbent 25 material. Adsorbent materials may also be packaged in membranes or bound to hemocompatible fibers.

Washing Methods

In another embodiment, residual compound or chemical product in the 30 treated blood product may be removed by "washing" the blood product by separating out the specific residual compound or chemical product, or a solution containing the residual compound or chemical product, from the desired components of the blood product and replacing any removed solution with a solution that is compatible with blood product storage and function prior to 35 transfusion. The present invention contemplates that any separation system compatible with human blood products would be acceptable for the methods of

the present invention. Blood separation techniques which are contemplated as appropriate technologies include those used in treatments such as blood-oxygenation, plasmapheresis, leukopheresis, hemopheresis, extracorporeal chemotherapy, hyperthermia, hypothermia, bone marrow transfusions, blood 5 transfusions processing by heart lung machines during surgery and dialysis for patients with kidney failure. There follows a list of separating technologies that may be applied in the present invention to wash blood products. These are presented as examples, and all patents and publications listed are hereby incorporated by reference. This list is not to be considered as limiting the 10 invention in any way, but is merely presented for guidance.

Field Flow Fractionating (FFF), is a chromatographic method for fractionating macromolecules and fine particles. A field acts perpendicular to flow through a narrow channel, forcing particles toward a wall. Simultaneously, diffusion tends to re disperse the particles. The particles least affected by the field, 15 and most dispersed by diffusion in the cross section move downstream most quickly. A pulse of a mixture dispersed or dissolved in a fluid carrier which flows steadily through a tube will thus emerge in several fractions or peaks, as in chromatography. Various types of external fields have been employed, yielding the following FFF subclasses: sedimentation (centrifugal), thermal, 20 thermogravitational, flow (pressure gradient causes cross flow through membrane walls), and concentration. The descriptions hereunder specifically address the following separation technologies: spinning membrane techniques, hollow fiber separators, centrifugal separation devices, separation techniques using flat membranes with tangential flow, and passive filtration techniques 25 relying on gravity flow to pass through a filter.

There are numerous membrane-moderated separation devices and methods known in the art. For example, U.S. Pat. No. 4,375,414 discloses immobilizing immunoactive materials on the side of a membrane on which the fluid being treated is flowing or having the material carried in fluid on the 30 opposite side of the membrane. U.S. Pat. No. 4,266,026 disclose use of an anisotropic membrane. U.S. Pat. No. 4,191,182 to Popovich, et al. describes a method and apparatus for plasmapheresis. Again, the system described employs a membrane with the appropriate pore sized to fractionate blood into cellular and plasma components:

35 A variety of spinning membrane techniques have been detailed in the literature. One such technique is embodied in the Autopheresis C plasma

collection device (available from Baxter Healthcare Corp., Deerfield, IL). Variables in using this device include the hematocrit swing across the device, the final (output) hematocrit, the flow rate of the blood, the rate of spinning of the membrane, the type of membrane material, and the size of the pores in the membrane.

5 Hollow fiber separators are described in U.S. Pat. No. 5,330,420. The patent describes membranes that can be used for the separation of blood products. The semi-permeable membrane described allows plasma solutions to pass through, but not red blood cells.

10 Centrifugal separation techniques are described in U.S. Pat. No. 3,705,100 which generally discusses a method and apparatus for fractionating blood in order to separate the blood components that may be desirable in blood transfusions. Taught is the use of centrifugal separation techniques and the use of a filtration membrane such as anisotropic and depth filter membranes. The 15 Haemonetics intraoperative blood salvage (autotransfusion) device, incorporating centrifugal separation techniques, is available from Terumo (Braintree, MA).

U.S. Pat. No. 4,350,156 describes separation techniques using flat membranes with tangential flow. Specifically, the patent discloses a continuous, 20 on-line system and apparatus for removing macromolecules from a physiological fluid such as blood. Membranes are employed in a blood flow path to separate blood plasma, cellular components, and macromolecules.

Passive filtration techniques relying on gravity flow to pass through a filter are also contemplated by the present invention for washing.

25 Hollow fiber separators are used with autotransfusion devices. The goal of these devices, hemoconcentration and small molecule removal/exchange, are applicable to the present invention. Other than the rate of spinning, the same variables apply to this technology. Hollow fiber membranes are commercially available from Mitsubishi.

30 Membrane compositions contemplated for washing methods of the present invention include, but are not limited to, nylon, polycarbonate and polyethersulfones. Membranes can be treated in various ways to increase or decrease hydrophilicity.

Monitoring the Removal Process

The fluorescent characteristics of some compounds of the present invention may be used to monitor the removal process. Measurement of fluorescence provides the opportunity for every treated sample to have a quality control check prior to transfusion to confirm treatment performance and completion. The present invention contemplates that an actinic light source with a low-pass filter <420 nm and a photodiode detector with a high pass filter >450 nm be placed in the waste stream so as to measure fluorescence. Fluorescence measurement is a reproducible way of determining whether the removal process is complete.

3. Decontamination of Vaccines and Other Biological Compositions

Vaccines and other biological compositions which are not derived from blood, such as recombinant DNA produced proteins and oligopeptide ligands, may also be decontaminated using methods of the present invention. Recombinant DNA produced proteins often are manufactured in large quantities in host organisms. Introduction of the decontamination compound may occur prior to amplification, so that as the host organisms grow, the compound is incorporated into the organism. Alternatively, the compound may be added after manufacture, but before the product is introduced into a mammal.

Removal of the compound before use may be desired here as well as with blood products for transfusion. Those methods mentioned above apply equally well in the case of vaccines and other biological compositions.

25 V. PRESERVATION OF BIOCHEMICAL PROPERTIES OF TREATED MATERIAL

When treating blood products to be used *in vivo*, one must ask whether the process or the compounds used alter the *in vivo* activity of the treated material. For example, red blood cell transfusion is a well established efficacious treatment for patients suffering large blood loss. However, if the inactivation treatment used greatly reduces the *in vivo* life of the red blood cells, then the treatment has no practical value. The compounds of the present invention are useful in inactivation procedures because the reaction can be carried out at temperatures compatible with retaining biochemical properties of blood and blood products. But not all methods of pathogen inactivation will inactivate

without significantly lowering the biological activity of the decontaminated material. Previously known compounds and protocols for inactivation have necessitated both exposure to light and the subsequent removal of molecular oxygen from the reaction before and during the exposure, to prevent damage to 5 blood products from oxygen radicals produced during irradiation. See L. Lin et al., Blood 74:517 (1989); US Patent No. 4,727,027, to Wiesehahn. The present invention may be used to decontaminate blood products without light, and in the presence of oxygen, without destroying the activity for which the products are prepared. Further, with methods of the present invention, there is no need 10 to reduce the concentration of molecular oxygen.

The present invention contemplates that activity of a blood product for *in vivo* use is not destroyed or significantly lowered if the blood product which is decontaminated by methods of the present invention retains the ability to function for the purpose it was prepared. By the same token, the activity of a 15 clinical sample is not destroyed or significantly lowered if the clinical sample which is decontaminated by methods of the present invention tests as would an untreated sample in common clinical chemistry tests, or if treatment results in variation from control values, if the variation is sufficiently predictable or consistent that the clinical test results may be effectively used in medical or 20 scientific analysis. In contrast, a blood product or clinical sample is considered to have incurred "significant damage" when the blood product no longer functions for the purpose it was prepared. For the purpose of the definition of "significant damage," the present invention contemplates that *in vitro* screens that are generally accepted in the scientific or medical field as accurately predictive of 25 blood product function are sufficient evidence to determine whether or not a blood product retains the ability to function for the purpose it was prepared.

VI. PREPARATION OF VACCINES

30 The preparation of viral vaccines is also contemplated by methods of the present invention. The present invention contemplates producing vaccines to a wide variety of viruses, including human viruses and animal viruses, such as canine, feline, bovine, porcine, equine and ovine viruses. The contemplated method is suitable for inactivating double stranded DNA viruses, single stranded 35 DNA viruses, double-stranded RNA viruses and single-stranded RNA viruses, including both enveloped and non-enveloped viruses. A contemplated method

for producing a vaccine for inoculation of a mammalian host susceptible to infection by a virus comprises growing culture of virus, isolated from an infected host, in a suitable mammalian cell culture, exposing at least one of the seed viruses to a compound having a nucleic acid binding ligand and a mustard group for a time sufficient to inactivate the virus to a non-infectious degree, under conditions which substantially preserve the antigenic characteristics of the inactivated viral particles, and combining said inactivated virus with a suitable adjuvant.

The inactivated virus may be formulated in a variety of ways for use as a vaccine. The concentration of the virus will generally be from about 10^6 to 10^9 plaque forming units (pfu)/ml, as determined prior to inactivation, with a total dosage of at least 10^5 plaque forming units per dose (pfu/dose), usually at least 10^6 pfu/dose, preferably at least 10^7 pfu/dose. The total dosage will usually be at or near about 10^9 pfu/dose, more usually being about 10^8 pfu/dose. The vaccine may include cells or may be cell-free. It may be an inert physiologically acceptable medium, such as ionized water, phosphate-buffered saline, saline, or the like, or may be administered in combination with a physiologically acceptable immunologic adjuvant, including but not limited to mineral oils, vegetable oils, mineral salts, and immunopotentiators, such as muramyl dipeptide. The vaccine may be administered subcutaneously, intramuscularly, intraperitoneally, orally, or nasally. Usually, a specific dosage at a specific site will range from about 0.1 ml to 4 ml, where the total dosage will range from about 0.5 ml to 8 ml. The number of injections and their temporal spacing may be highly variable, but usually 1 to 3 injections at 1, 2 or 3 week intervals are effective.

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EXPERIMENTAL

The following examples serve to illustrate certain preferred embodiments and aspects of the present invention and are not to be construed as limiting the scope thereof.

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In the experimental disclosure which follows, the following abbreviations apply: eq (equivalents); M (Molar); μ M (micromolar); N (Normal); mol (moles); mmol (millimoles); μ mol (micromoles); nmol (nanomoles); gm (grams); mg (milligrams); μ g (micrograms); L (liters); ml (milliliters); μ l (microliters); cm (centimeters); mm (millimeters); μ m (micrometers); nm (nanometers); °C (degrees Centigrade); HPLC (High Pressure Liquid Chromatography); Q (quinacrine); QM (quinacrine mustard); DMSO (dimethylsulfoxide); Htc

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(hematocrit); RBC (red blood cell); LB (Luria Broth); N-acetyl-cysteine (NAC); BUN (blood urea nitrogen); Creat. (creatinine); phos acid (phosphoric acid); alk (alkaline phosphatase); ALT (Alanine Aminotransferase); AST (Aspartate Transaminase); LDH (lactate dehydrogenase); GGT (Glutamate Transferase); cfu (culture forming units); pfu (plaque forming units); DMEM (Delbecco's modified eagles medium); FBS (fetal bovine serum); PRBC (packed red blood cells); PCR (polymerase chain reaction); rpm (revolutions per minute); TC (tissue culture); NHSP (normal human serum pool); LSM (lymphocyte separation medium); NCS (newborn Calf Serum); PBS (phosphate buffered saline).

10 While it is available commercially from Baxter Healthcare Corp., Deerfield, IL, Adsol used in the following experiments was made by sterile filtering the following mixture: 22 g glucose, 9 g NaCl, 7.5 g mannitol, and .27 g adenine in 1 liter of distilled H2O.

15 The polymerase chain reaction (PCR) is used in some of the examples below. PCR is a method for increasing the concentration of a segment of a target sequence in a mixture of genomic DNA without cloning or purification. See K.B. Mullis *et al.*, U.S. Patents Nos. 4,683,195 and 4,683,202, hereby incorporated by reference. This process for amplifying the target sequence consists of introducing a large excess of two oligonucleotide primers to the DNA mixture 20 containing the desired target sequence, followed by a precise sequence of thermal cycling in the presence of a DNA polymerase. The two primers are complementary to their respective strands of the double stranded target sequence. To effect amplification, the mixture is denatured and the primers then are annealed to their complementary sequences within the target molecule.

25 Following annealing, the primers are extended with a polymerase so as to form a new pair of complementary strands. The steps of denaturation, primer annealing, and polymerase extension can be repeated many times (i.e. denaturation, annealing and extension constitute one "cycle;" there can be numerous "cycles") to obtain a high concentration of an amplified segment of the desired target sequence. The length of the amplified segment of the desired 30 target sequence is determined by the relative positions of the primers with respect to each other, and therefore, this length is a controllable parameter. By virtue of the repeating aspect of the process, the method is referred to by the inventors as the "polymerase chain reaction".

EXAMPLE 1

This example measures the R17 inactivation activity of quinacrine mustard (QM) solutions made in either Adsol or DMSO. The bacteriophage R17 has a single stranded RNA genome of approximately 1.2×10^6 daltons, and is 5 difficult to inactivate compared to many other targets. See generally L. Lin *et al.*, Blood 74:517 (1989). The advantage of the R17 system is that inactivation can be readily assayed in the laboratory.

The assay used to determine inactivation measures the ability of the phage to subsequently infect bacteria and inhibit their growth. The phage was grown 10 up in Hrf 3000 bacteria. (R17 and Hrf 3000 were obtained from American Tissue Culture Collection (ATCC), Washington, D.C.). First, the R17 stock virus was diluted (10.9 logs/ml in LB broth) 1:20 in Adsol (R17-Adsol). Then a 30% hematocrit (Htc) red blood cell concentrate in R17-Adsol mixture was prepared by spinning down red blood cells (RBC) from whole blood and resuspending 3.5 ml 15 RBC pellet in 7.0 ml R17-Adsol. In this, and the following experiments, Htc was measured on a Model F800 Sysmex cell counter (Toa Medical Electronics, Kobe, Japan). Ten 1 ml aliquots of the samples were then transferred to sterile tubes.

Approximately 2 mg of QM, commercially available from Aldrich, Inc., Milwaukee, WI, was weighed out into each of two tubes. Samples were then 20 dissolved in DMSO or Adsol, respectively, to a final concentration of 0.4 mg/ml. QM in Adsol is a suspension, not a solution, at this concentration.

Next, the QM suspension was added to the R17-Adsol samples to achieve 25 the following final concentrations of QM in the sample tubes: 2.5, 5.0, 10, or 20 $\mu\text{g}/\text{ml}$. The QM was completely solubilized at these concentrations. Positive control samples were also prepared, where 50 μl of either Adsol or DMSO was added to R17-Adsol samples. The samples were allowed to stand at room 30 temperature for at least 1 hour. Then the samples were titered by an R17 phage assay. Sterile 13 ml dilution tubes were prepared with LB broth. To make the dilutions, a 0.1 ml aliquot of the solution of phage was added to the first dilution tube of 0.4 ml of media. Then 0.02 ml of this solution was added to the second tube of 0.5 ml media (1:25). The second solution was then diluted serially (1:25) into the remaining tubes. To each diluted sample was added 0.05 ml of Hrf 3000 bacteria cultured overnight and 3 ml of molten LB top agar. The mixed materials 35 were poured onto LB broth plates. After the top agar hardened, the plates were incubated at 37 °C overnight. Plaques were counted the following morning and

the titer of the phage remaining after treatment was calculated based on the dilution factors.

The results are shown in Table 2, below, and FIG. 1. It is clear from the data that even at concentrations as low as 2.5 μ g/ml QM is effective in 5 inactivating R17. At concentrations above 10 μ g/ml, complete inactivation is achieved, to the limit of detection of this assay.

TABLE 2

Sample #	(QM) (μ g/ml)	Solvent	Log Titer
1	0	Adsol	9.8
2	0	DMSO	9.8
3	2.5	Adsol	3.25
4	5	Adsol	3.55
5	10	Adsol	1.0
6	20	Adsol	1.3
7	2.5	DMSO	5.2
8	5	DMSO	2.3
9	10	DMSO	2.4
10	20	DMSO	1.0

EXAMPLE 2

10 The purpose of this example is to show that the presence of RBC does not significantly effect R17 inactivation by compounds and methods of the present invention. Two different compounds were tested, QM and Compound 1, the synthesis of which is described in Example 17, below. For QM, the procedure was as follows: first, approximately 60% Htc RBC concentrate was prepared by 15 dilution in Adsol. The sample was again diluted with Adsol into sterile tubes to give RBC concentrate with a final Htc of 2%, 6%, 20% or 60% (0.5 ml final volume in each tube).

Next, an R17 stock (11.3 logs/ml in LB) was diluted 1:10 in Adsol (R17-Adsol). This stock was added (0.5 ml) to each tube to give a final Htc of 20 approximately 1%, 3%, 10% or 30% in 1 ml. A positive control sample was prepared without RBC by combining 0.5 ml of R17-Adsol with 0.5 ml Adsol. QM (3.4 mg) was dissolved in H₂O to reach a final concentration of 0.1 mg/ml. Then a 10 μ l aliquot of the QM solution was added to each R17 sample and the samples were incubated approximately 2 hours. A negative control was not treated with

QM. The samples were then titered in an R17 phage assay, as described in Example 1, above.

The results are shown in Table 3 and FIG. 2. It is clear from the data that QM inactivates R17 in all of the Htc tested.

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TABLE 3

Sample #	Htc (%)	QM (μg/ml)	Log Titer
1	0	0	8.8
9	0	1.0	2.0
10	1	1.0	4.0
11	3	1.0	2.1
12	10	1.0	2.6
13	30	1.0	2.4

Another experiment was performed to test the inactivation ability of a novel compound, Compound 1. A 1:1000 dilution of R17 (stock titer was 11.9 logs) was prepared in 25 ml packed red blood cells. To each of 5 tubes was added 5 ml of this R17-packed red blood cell solution. Compound 1 was then dissolved in saline to a final concentration of 3 mg/ml. The compound in solution was added to the 4 tubes as follows: the first tube, the control tube, received saline only; the second tube received 10 μg/ml of Compound 1 in saline; the third tube received 30 μg/ml of Compound 1 in saline; the forth tube received 100 μg/ml Compound 1 in saline and the final tube received 300 μg/ml of Compound 1 in saline. The tubes were mixed and then incubated at 4°C overnight. The results showed R17 inactivation activity. Concentrations above 30 μg/ml inactivated approximately 4 logs of R17 with a starting titer of 10 logs of R17.

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EXAMPLE 3

This example sets forth the kinetics of R17 inactivation by QM. To measure the kinetics of inactivation, reactive QM must be quenched so that intermediate time points provide a reliable measure of the R17 inactivation at a particular time. Two methods were used here in combination to quench the reaction. First, NAC was added to samples to react with excess QM. Second, samples were rapidly diluted into LB medium to reduce the effective QM concentration in the sample. The control experiments described below

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demonstrate that this dual approach does effectively quench residual QM, allowing for a valid measure of the reaction kinetics to be taken.

Samples were prepared in the following manner. A dilution of R17 (1:20) into Adsol was prepared: 0.15 ml phage (11.3 logs/ml) + 2.85 ml Adsol. An aliquot of sterile-filtered 0.1 M NAC was thawed for use to quench the QM reaction with R17.

Tubes were then prepared for standard dilution of phage, containing appropriate volumes of LB.

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TABLE 4

Sample #	Treatment
1	QM first, NAC quench at 0 min., dilute
2	QM first, NAC quench at 2 min., dilute
3	QM first, NAC quench at 4 min, dilute
4	QM first, NAC quench at 8 min, dilute
5	QM first, NAC quench at 16 min, dilute
6	QM first, NAC quench at 32 min, dilute
7	QM first, NAC quench at 64 min, dilute
8	QM first, NAC quench at 128 min, dilute
9	first add NAC, then QM, dilute
10	first add NAC, then QM, dilute at end
11	add NAC / no QM, dilute
12	add NAC / no QM, dilute at end
13	no NAC / no QM, dilute at end

A set of tubes were prepared, herein called quenching tubes, containing quenching factors (NAC and/or dilution with LB), to receive the samples at appropriate time points. Cysteine (44 μ l aliquots) was added to quenching tubes numbered 1-12.

QM (1.5 mg) was dissolved in Adsol (25.0 ml) to a final concentration of 0.1 mg/ml. Then the QM solution was diluted 100x into Adsol: 50 μ l QM solution + 4.95 ml Adsol; 1 μ g/ml final concentration.

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Table 4 sets forth how each control and experimental sample was treated.

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The controls were treated first, by placing aliquots (100 μ l) of phage into quenching tubes 9-13, then immediately adding 100 μ l of 1 μ g/ml QM to quenching tubes 9 and 10 and 200 μ l Adsol to quenching tubes 11 and 12. Adsol

(250 μ l) was added to quenching tube 13. Then samples 9 and 11 were diluted into LB broth for phage assay.

The experimental samples were treated next. Phage (1.0 ml) was removed into a sterile 15 ml tube. QM (1.0 ml, 1.0 μ g/ml) was added. This mixture was removed (by 200 μ l aliquots) into quenching tubes 1-8 at the following times: 0, 2, 4, 8, 16, 32, 64, and 128 minutes. The samples were mixed and immediately diluted into LB broth for phage assay. Finally, samples 10, 12, and 13 were diluted into LB broth for phage assay.

Results are shown in Table 5 and FIG. 3. While NAC alone does not kill R17 (compare samples #11 and #12 with sample #13), when added before QM, NAC provided a substantial, but not complete protection against QM inactivation (compare samples #7 and #10). The combination of NAC and dilution resulted in almost complete quenching of QM activity (compare samples #1 and #13). QM inactivation of R17 was complete within 2 hours.

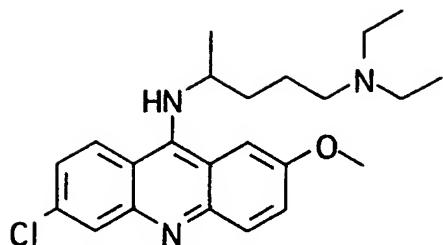
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TABLE 5

Sample	1	2	3	4	5	6	7	8	9	10	11	12	13
Log Titer	8.11	6.81	5.79	4.10	3.74	2.69	2.39	\leq 2.4	8.66	6.32	8.73	8.35	8.80

EXAMPLE 4

This experiment measures the loss of QM activity upon pre-incubation of drug in Adsol. It is believed that mustards react by thermally allowed pathways. They can be hydrolyzed in aqueous solution. This experiment was designed to measure loss of QM anti-viral activity in a particular aqueous solution, Adsol. Previous results have shown that QM anti-viral activity did not decrease rapidly upon pre-incubation of the drug in Adsol. (Results not shown). A concern in those experiments was the possibility of light-dependent inactivation, because samples were diluted into LB without making extraordinary efforts to shield ambient light, and because acridines are known to inactivate by photodynamic effects. This experiment was repeated under conditions where ambient light levels were carefully controlled throughout the experiment, in order to exclude the possibility that R17 inactivation was due to light-mediated effects. Also, additional controls were added to examine the effects of light in samples that were deliberately exposed to room lights and to examine the inactivation by the parent compound, quinacrine, the structure of which follows:



The following procedure was performed in a biosafety cabinet without lights. R17 phage (10.9 logs/ml) was diluted 10 fold into Adsol: 1.0 ml R17 stock
 5 + 9.0 ml Adsol. One (1) ml of the diluted phage was transferred into ten sterile 1.5 ml tubes. A 0.1 mg/ml QM solution was prepared by dissolving 3.4 mg QM (weighed in hood) with 34 ml H₂O. The resulting solution was wrapped in foil to shield from light. Then, 10 μ l QM was added to tubes after 0, 10, 40, 60, 120 or 240 minutes of pre-incubation. Samples were again wrapped in foil to prevent
 10 exposure to light. For a light control, 10 μ l QM was added to 1.0 ml phage at time 0, and the sample was not wrapped in foil. A 1 mg/ml solution of quinacrine in DMSO was prepared as another control. One (1) μ l of this was added to each of two samples. Then one sample was incubated in foil (sample Q) and one without foil (sample Q + light). All samples were incubated for 2 hours 15
 15 minutes beyond final addition of QM. Total incubation for the time zero sample was 6 hours 15 minutes.

The following work was performed with very low ambient light (source was one closed doorway): bacteria was diluted and plated in the dark. For the light positive controls, the samples were exposed to ambient light during dilutions, then moved to dim lighting conditions during plating.

The results are shown in FIG. 4 and Table 6. From these results it is clear that there was no light-dependent kill by QM or quinacrine under the conditions of this experiment. Further, QM activity was not diminished after a 4 hour pre-incubation in Adsol.

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TABLE 6

Sample	Control	QM + light	Q	Q + light	0 min	10 min	40 min	1 hour	2 hour	4 hour
Log Titer	9.7	3.9	9.7	9.7	3.8	3.9	3.9	4.0	4.8	4.0

EXAMPLE 5

QM activity was not diminished after a 4 hour pre-incubation in Adsol, as shown by Example 4, above. A goal of this example is to determine whether QM is inactivated more rapidly by pre-incubation in the presence of red cells. This 5 example also examines the kinetics of removal of QM from red blood cell solutions by an adsorbent material, to establish the effectiveness of a removal technique in removing compounds containing a mustard group.

First, phage dilutions were prepared. R17 (11.3 log/ml stock) was diluted 1:10 into Adsol: 0.7 ml phage + 6.3 ml Adsol. Diluted phage (0.5 ml) was placed 10 into 15 sterile 1.5 ml tubes labeled 1-15. The treatment for each tube is shown in Table 7.

TABLE 7

Sample #	Treatment	Time (min)
1	none	--
2	QM-adsol	0
3	QM-adsol	240
4	QM-RBC	0
5	QM-RBC	15
6	QM-RBC	30
7	QM-RBC	60
8	QM-RBC	120
9	QM-RBC	240
10	QM-XAD	0
11	QM-XAD	15
12	QM-XAD	30
13	QM-XAD	60
14	QM-XAD	120
15	QM-XAD	240

15 Next, QM solutions were prepared. Approximately 20 ml of packed red blood cells (PRBC) were spun down in a 50 ml conical tube at 1600 rpm for 9 minutes. The volume of the pellet after spinning was 17 ml. Approximately 3 mg QM was weighed out on a weighing paper in a biosafety cabinet (actual weight was 4.5 mg). The sample was then transferred to a 50 ml conical tube. The sample was

dissolved in Adsol to a concentration of 0.1 mg/ml (actual volume of Adsol was 45 ml). Next, the red blood cell pellet was diluted 1:1 with 17 ml of the QM solution. The tube contents were mixed gently by inversion several times. This is subsequently called the QM-RBC solution.

5 Amberlite XAD 16™ (a commercially available adsorbent from Sigma, St. Louis, MO) was weighed out (0.452 g) and transferred to a 15 ml conical tube. An aliquot of the QM-RBC solution (9 ml) was transferred to the 15 ml tube containing 0.5 g XAD-16 and mixed gently by inversion. This is subsequently referred to as the QM-XAD solution. The QM solution was diluted with an equal
10 volume of Adsol (1 ml of each). This is subsequently referred to as the QM-Adsol solution.

15 At each time point, 0.1 ml was removed from QM-RBC, QM-XAD and QM-Adsol into a 1.5 ml Eppendorf tube. The tubes' contents were spun down 10 sec at full speed in a microfuge to pellet cells and resin. Then 5 µl aqueous phase containing QM was transferred to the appropriate tube containing phage. The phage containing QM was then incubated in the dark. Finally, dilutions were made and the phage were plated.

TABLE 8

Sample	Log Titer
1	9.7
2	2.9
3	3.7
4	2.8
5	3.4
6	5.0
7	7.1
8	8.0
9	9.7
10	3.2
11	7.9
12	9.1
13	9.6
14	9.8
15	9.8

The results appear in Table 8, above, and FIG. 5. QM anti-viral activity was removed upon a 4 hour pre-incubation with red blood cells. The adsorbent removal material, Amberlite XAD-16™, also removed QM from blood within 1 hour. These results suggest that either incubation in the presence of red blood cells or treatment with an adsorbent resin, or the two treatments combined, will be sufficient to rapidly remove residual QM after inactivation.

EXAMPLE 6

10 The purpose of this example is to measure inactivation of duck hepatitis B virus (DHBV) by a method of the present invention. DHBV was chosen as a model for human hepatitis B virus because of the similarities in design between the two viruses. See Ganem, D. and Varmus, H. "The Molecular Biology of the Hepatitis B Viruses," Ann. Rev. Biochem. 56:651 (1987).

15 Infected duck hepatocytes were prepared as follows. Duck hepatocytes were isolated from the livers of approximately 1 week old ducklings. Ducklings were prescreened and found negative for DHBV. Each of the ducklings was anesthetized, then infused with 0.5 ml sodium heparin via the portal vein. Next, each duckling was perfused with 75 ml of a solution containing 200 ml 1X
20 MEM/Earle's BSS + 2 ml Hepes buffer + 2 ml of 1% EGTA (in 1X MEM). Then, the ducklings were perfused for 20 minutes with a filter sterilized solution containing 30 mg of Collagenase A (commercially available from Boehringer-Mannheim Biochem., Indianapolis, IN) + 200 ml Ham's F-12/DMEM medium.

At this point, the liver was removed, cut up into a fine mush and placed
25 in a 125 ml bottle containing 50 ml Ham's F-12/DMEM. Approximately 10 ml of a solution containing 5 mg DNase I and 25 ml Ham's F-12/DMEM was added to the liver suspension. The suspension was spun at 200 rpm for 10 minutes.

The suspension was then strained through gauze pads, the 125 ml bottle was rinsed with the remaining 15 ml of the DNase I solution and the rinsing was
30 also strained into the liver suspension. The cell suspension was equally divided into 2 x 50 ml centrifuge tubes and pelleted at 50 x g for 2 minutes. The pellets were resuspended in 10 ml of a solution containing Medium 199/Earle's BSS, 5% calf serum and pelleted. This process was repeated two more times. The third pelleting was resuspended in 10 ml plating medium. Another 10 ml plating
35 medium was added to each tube.

The liver cell suspension was filtered through a 70 micron cell strainer into a 50 ml centrifuge tube. Aliquots of the cell suspension (approximately 0.5 ml) were transferred to petri dishes containing 2 ml plating medium, to obtain a level of confluence corresponding to approximately 6 to 8×10^6 viable cells per 5 petri dish. After a two hour incubation at 37°C, the medium was changed to L-15 medium (commercially available from Gibco, Grand Island, NY) (containing 0.9 g/L galactose, 0.55 g/L Na pyruvate)/DMSO. The medium was again changed at 24 hours and every 48 hours thereafter. Cells were grown in culture for 5-7 days.

Next, viral inactivation was performed. DHBV stock virus was thawed at 10 37°C for 15 min in an oven. The virus was then spun down at 14000 rpm in a microfuge for 5 min at room temperature and the supernatant was transferred to a fresh tube, avoiding material at the bottom of the tube. The spin and transfer were repeated and the samples were placed on ice.

15 Approximately 7 ml of human whole blood was drawn into a tube containing acid citrate dextrose anticoagulant. The cells were spun down at 1600 rpm for 9 minutes to pack the red cells. The plasma was withdrawn and replaced with an equal volume of Adsol (2.9 ml).

The virus was diluted 0.25 ml into 2.25 ml red blood cells and the mixture was vortexed to create a 10^{-1} dilution. The diluted virus was then aliquoted in 20 sterile tubes as follows: 50 μ l as an untreated sample; 1.8 ml to be treated with 40 μ g/ml QM; and 0.5 ml to be treated with 10 μ g/ml QM. Next, a 1 mg/ml QM solution was prepared by dissolving 3.2 mg QM in 3.2 ml sterile ddH₂O. Aliquots of the QM solution were added to the tubes containing virus, as follows: 72 μ l QM was added to the 1.8 ml sample to achieve a final concentration of QM of 25 approximately 40 μ g/ml and 5 μ l QM was added to the 0.5 ml sample to achieve a final concentration of QM of 10 μ g/ml. The samples were incubated for 4 hours at room temperature.

After incubation the red blood cells were spun down in a microfuge. Plasma/Adsol supernatant was removed. Dilutions of each sample were 30 prepared by serial dilution of 100 μ l virus into 0.9 ml PBS/10% NCS (PBS was 10 mM phosphate, approximately 150 mM NaCl, pH 7.4). The untreated control (sample #1) was diluted to 10^{-7} . The treated samples (#2 and #3) was diluted to 10^{-4} .

Plates containing liver suspension were then inoculated according to the 35 scheme set forth in Table 9. Plates were inoculated with approximately 100 μ l virus in duplicate (see below) and cultured for 1, 10, or 15 days. Samples were

then analyzed by PCR and by slot blot hybridization to confirm the presence of viral DNA.

Slot blot hybridization was performed for all of the samples after harvesting DNA from tissue culture samples. PCR analysis was performed on 5 selected samples. Samples were denatured with 3M NaOH, as were plasmid pD1.5G DNA samples for labeling. Samples were then neutralized with NH₄OAc. 400 μ l of 1M NH₄OAc was added to each well of a Mini Fold II Slot Blot Apparatus, commercially available from VWR Scientific, Greenbelt, MO, fitted with a filter, as were aliquots of each sample. Vacuum was applied to the 10 apparatus until all samples had been pulled through the filter. The filter was then baked to dry. Next, the filter was pre-hybridized in a mixture of 250 ml of 20X SSC (175.3 g NaCl, 88.2 g Na citrate in 800 ml H₂O), 50 ml of 50X Denhardt's solution (5g Ficoll, available from Sigma, St. Louis, MO, 5g polyvinylpyrrolidone, and 5 g bovine serum albumin with 500 ml H₂O), 20 ml of mg/ml denatured 15 salmon sperm DNA, 180 ml H₂O, 500 ml formamide and 10 ml of 10% solution of sodium dodecyl sulfate in H₂O. Probe was prepared as follows: 3 μ l of pD1.5G (67 ng/ μ l) and 5 μ l of 15 ng/ μ l random hexamer oligonucleotides were heated and cooled again, then 4 μ l of 5X labeling buffer, 2 μ l of dGAT mixture (5 mM each of dGTP, dATP, dTTP, in TE), 1 μ l of Klenow, and 5 μ l of [α ³²P]dCTP was 20 added and incubated. Reaction was stopped by adding 25 mM EDTA. Then 5 X 10⁵ counts per minute of probe per ml of hybridization solution was added to the filter and allowed to hybridize overnight. The filter was removed, and low stringency wash solution (50 ml of 20X SSC, 940 ml of H₂O, and 10 ml of 10% SDS) was added to cover the filter for a wash during shaking, which was repeated 25 2 times, the last time adding high stringency wash solution (5 ml of 20X SSC, 990 ml of H₂O, and 10 ml of 10% SDS) instead. The filter was then exposed to film to obtain an appropriate exposure, and the film was then scored for positive hybridization. A negative control sample containing calf thymus DNA was also run. Table 10 summarizes PCR and slot blot hybridization data. (NP signifies 30 that PCR was "not performed" for that sample. A plus sign signifies that DHBV nucleic acid was amplified in PCR. A minus sign signifies that it was not amplified).

TABLE 9

Sample	Treatment	Dilution	Incubation
1	no virus	NA	10 days
2	untreated	10^{-7}	10, 15
3	untreated	10^{-6}	10, 15
4	untreated	10^{-5}	10, 15
5	untreated	10^{-4}	10, 15
6	untreated	10^{-3}	10, 15
7	40 μ g/ml QM	10^{-5}	10, 15
8	40 μ g/ml QM	10^{-4}	10, 15
9	40 μ g/ml QM	10^{-3}	10, 15
10	40 μ g/ml QM	10^{-2}	10, 15
11	40 μ g/ml QM	10^{-1}	1, 10, 15
12	10 μ g/ml QM	10^{-5}	10, 15
13	10 μ g/ml QM	10^{-4}	10, 15
14	10 μ g/ml QM	10^{-3}	10, 15
15	10 μ g/ml QM	10^{-2}	1, 10, 15

Referring to Table 10, viral titer was 6 logs per ml based on PCR positive signal for plate #9. A dose of 10 μ g/ml QM inactivated 4 logs per ml based on PCR positive signal for plates #60 and #61. A dose of 40 μ g/ml QM inactivated >5 logs of DHBV per ml based on the absence of a PCR signal and slot blot signals in all samples tested.

TABLE 10

Sample #	Incubation (days)	Plate #'s	Blot Results	PCR Results
1	10	1*,2	--,--	-
2	10	3,4	--,--	NP
2	15	5*,6	--,--	-
3	10	7,8	--,--	NP
3	15	9*,10	--,--	+
4	10	11*,12	--,--	-
4	15	13*,14	±,+	+
5	10	15*,16*	+,--	+,+
5	15	17*,18	++,+	+
6	10	19,20*	++,+	+
6	15	21*,22	++,+	+
7	10	23,24	--,--	NP
7	15	25,26	--,--	NP
8	10	27,28	--,--	NP
8	15	29,30	--,--	NP
9	10	31*,32	--,--	-
9	15	33*,34	--,--	-
10	10	35*,36	--,--	-
10	15	37*,38	--,--	-
11	1	39,40*	--,--	-
11	10	41*,42	--,--	-
11	15	43*,44	--,--	-
12	10	45,46	--,--	NP
12	15	47,48	--,--	NP
13	10	49,50*	--,--	-
13	15	51,52	--,--	NP
14	10	53*,54*	--,--	--,--
14	15	55,56*	--,--	-
15	1	57*,58	--,--	-
15	10	59,60*	--,--	+
15	15	61*,62	--,--	+

* These plates were tested in PCR. Results appear in PCR column.

EXAMPLE 7

The purpose of this example is to measure inactivation of cell-free HIV by QM. As in the R17 assay, small aliquots of QM were added to stock HIV-1. The stock QM solution was prepared by dissolving 3.4 mg of the compound in tissue culture media (DMEM/15% FBS) to reach a final concentration of 0.6 mg/ml of QM. The QM was a colloidal suspension rather than a solution at this concentration, which was used in the experiment. Stock HIV ($10^{4.2}$ plaque forming units/ml) was in DMEM/15% FBS. QM solution was added to aliquots of stock HIV-1 to obtain a final total sample volume of 0.5 ml, having the following final concentrations of QM: 3 μ g/ml, 10 μ g/ml, or 30 μ g/ml. The 0.5 ml test aliquots were placed in 24 well polystyrene tissue culture plates. Two controls were prepared, one containing HIV-1 stock only, and one containing QM without HIV-1 stock. All samples were incubated for one hour at room temperature, then stored at -70° C until assayed for infectivity by a microtiter plaque assay. Aliquots for measurement of residual HIV infectivity in the samples treated with a compound of the present invention were withdrawn and cultured.

Residual HIV infectivity was assayed using an MT-2 infectivity assay. (Previously described in Hanson, C.V., Crowford-Miksza, L. and Sheppard, H.W., J. Clin. Micro 28:2030 (1990)). The assay medium was 85% DMEM (with a high glucose concentration) containing 200 μ g of streptomycin, 200 U of penicillin, 50 μ g of gentamicin, and 1 μ g of amphotericin B per ml, 15% FBS and 2 μ g of Polybrene (Sigma Chemical Co., St. Louis, Mo.) per ml. Test and control samples from the inactivation procedure were diluted in 50% assay medium and 50% normal human pooled plasma. The samples were serially diluted in 96-well plates (Corning Glass Works, Corning, N.Y.). The plates were incubated at 37°C in a 5% CO₂ atmosphere for 1 to 18 hours. MT-2 cells (0.025 ml) [clone alpha-4, available (catalog number 237) from the National Institutes of Health AIDS Research and Reference Reagent Program, Rockville, Md.] were added to each well to give a concentration of 80,000 cells per well. After an additional 1 hour of incubation at 37°C in 5% CO₂, 0.075 ml of assay medium containing 1.6% SeaPlaque agarose (FMC Bioproducts, Rockland, Maine), prewarmed to 38.5°C was added to each well. The plates were kept at 37°C for a few minutes until several plates had accumulated and then centrifuged in plate carriers at 600 x g for 20 minutes. In the centrifuge, cell monolayers formed prior to gelling of the agarose layer. The plates were incubated for 6 days at 37°C in 5% CO₂ and stained

5 by the addition of 0.05 ml of 50 μ g/ml propidium iodide (Sigma Chemical Co.) in phosphate-buffered saline (pH 7.4) to each well. After 24 to 48 hours, the pink/orange fluorescence-stained microplaques were visualized by placing the plates on an 8,000 μ W/cm² 304 nm UV light box (Fotodyne, Inc., New Berlin, Wis.). The plaques were counted at a magnification of 20x to 25x through a stereomicroscope.

TABLE 11

Sample	Log Titer
no QM	4.2
3 μ g/ml QM	3.4
10 μ g/ml QM	2.0
30 μ g/ml QM	<1.7

10 The results appear in Table 11, above. At a concentration of 30 μ g/ml, QM was able to inactivate cell-free HIV completely to the level of detection of the plaque assay used.

EXAMPLE 8

15 The last example demonstrated that QM was able to inactivate cell free HIV. HIV can also be found within certain types of cells. This example examines the ability of QM, at varying concentrations, to inactivate the cell-associated form of HIV.

10 H9 cells chronically infected with HIVIIIB were used. (H9/HTLV-III-B NIH 1983 Cat.#400). Cultures of these cells were maintained in high glucose Dulbecco Modified Eagle Medium supplemented with 2 mM L-glutamine, 200 units/ml penicillin, 200 μ g/ml streptomycin, and 9% fetal bovine serum (Intergen Company, Purchase, N.Y.) For maintenance, the culture was split once a week, to a density of 3×10^5 to 4×10^5 cells/ml. About four days after splitting, 25 8.8% sodium bicarbonate was added as needed. For the inactivation procedure, the cells were used three days after they were split. They were spun from their culture medium at 400 g for 10 minutes, the supernatant was discarded, and the cells were resuspended in approximately 8 ml of 85% DMEM + 15% FBS, to a concentration of 2×10^6 cells/ml. Aliquots (1 ml) of the infected cell suspension 30 were placed in 15 ml tubes for QM free controls and for the QM experimental sample. A stock solution of QM (1 mg/ml in sterile ddH₂O) was diluted into the

15 ml tubes in the appropriate aliquots to yield a final concentration of either 0, 3, 10, 30, 100, or 150 μ g/ml. The samples were incubated for two hours, with periodic thorough mixing, then stored at -80° C until analyzed by microtiter plaque assay.

5 The stored samples were thawed at 37° C, then titrated in an HIV microtiter plaque assay, as described in Hanson, C.V., Crawford-Miksza, L. and Sheppard, H.W., J. Clin. Micro 28:2030 (1990), and as described in Example 7, above, with the following modifications. The samples were serially diluted directly in 96-well plates (Corning Glass Works, Corning, N.Y.). The plates were
10 incubated at 37°C in a 5% CO₂ atmosphere for 1 to 18 hours. MT-2 cells (0.025 mL) [clone alpha-4, available (catalog number 237) from the National Institutes of Health AIDS Research and Reference Reagent Program, Rockville, Md.] were added to each well to give a concentration of 80,000 cells per well. After an additional 1 hour of incubation at 37°C in 5% CO₂, 0.075 mL of assay medium
15 containing 1.6% SeaPlaque agarose (FMC Bioproducts, Rockland, Maine), prewarmed to 38.5°C was added to each well. The plates were kept at 37°C for a few minutes until several plates had accumulated and then centrifuged in plate carriers at 600 x g for 20 minutes. In the centrifuge, cell monolayers formed prior to gelling of the agarose layer. The plates were incubated for 6 days at 37°C in 5% CO₂ and stained by the addition of 0.05 mL of 50 μ g/mL propidium iodide
20 (Sigma Chemical Co.) in phosphate-buffered saline (pH 7.4) to each well. After 24 to 48 hours, the pink/orange fluorescence-stained microplaques were visualized by placing the plates on an 8,000 μ W/cm² 304 nm UV light box (Fotodyne, Inc., New Berlin, Wis.). The plaques were counted at a magnification of between 20X
25 and 25X through a stereomicroscope.

The results appear in Table 12.

TABLE 12

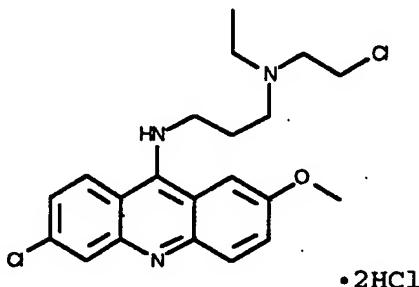
Sample	Log Titer	Log Reduction
no QM	5.5	-
3 μ g/ml	2.7	-2.8
10 μ g/ml	<0.7	>-4.8
30 μ g/ml	<0.3	>-5.2
100 μ g/ml	1.75	-3.75
150 μ g/ml	<0.3	>-5.2

It is clear from this example that QM inactivates cell-associated HIV, even at very low concentrations such as 10 μ g/ml and below.

5

EXAMPLE 9

This example sets forth the ability of two compounds having a nucleic acid binding ligand and a mustard group, QM, and N-(2-chloroethyl)-N-ethyl-N'-(6-chloro-2-methoxy-9-acridinyl)-1,3-propanediamine dihydrochloride ("ICR-170") (commercially available from Polysciences Inc, Warrington, PA) to inactivate 10 both cell-free and cell-associated HIV in the presence of red blood cells. The structure of ICR-170 is shown below.



15

For the cell free HIV inactivation, 15 ml of PRBC was mixed with 5 ml Adsol for a final volume of 20 ml. Then ten 2 ml aliquots were added to 15 ml conical tubes. Varying doses of the two compounds were next added to the tubes. The stock compound solutions were both 1 mg/ml in saline, stored at 4 °C. ICR-170 was in solution at this concentration. The following volumes of the two test 20 compounds were added to the PRBC tubes: 20, 40, 80 or 160 μ l; to produce final concentrations of the test compound of 10, 20, 40, or 80 μ g/ml.

25

After addition of the compounds, the samples were incubated for 100 minutes at room temperature in the dark, with mixing every 30 minutes. Subsequently, the red blood cells were pelleted by spinning for 5 minutes at 2500 rpm. The supernatant was removed and NHSP was added so that the sample contained 15% NHSP. Samples were stored at -80°C.

30

Inactivation of cell-associated HIV was performed in a similar manner, with the following exceptions. H9 cells chronically infected with HIVIIIB were used. (H9/HTLV-III-B NIH 1983 Cat.#400). Cultures of these cells were maintained in high glucose DMEM supplemented with 2 mM L-glutamine, 200 units/mL penicillin, 200 μ g/ml streptomycin, and 9% fetal bovine serum

(Intergen Company, Purchase, N.Y.) For maintenance, the culture was split once a week, to a density of 3×10^5 to 4×10^5 cells/ml and about four days after splitting, 3.3% sodium bicarbonate was added as needed. For the inactivation procedure, the cells were used three days after they were split. The cells in a sample of this stock were counted on a Neubauer type Hemacytometer (commercially available from VWR Scientific, Greenbelt, MO), and found to have 1.07×10^6 cells/ml. An aliquot (18.7 ml, 20×10^6 cells) was pelleted and resuspended in 5 ml Adsol. This 5 ml of cell suspension was then added to 15 ml of PRBC. The sample was divided and compound was added as described above for the cell-free samples. The samples were incubated for 100 minutes, followed by the addition of 3 ml of a 1:1 mixture of NHSP and RPMI-1640 (commercially available from Irvine Scientific, Santa Ana, CA). Next, each sample was placed in a 15 ml tube containing 6 ml lymphocyte separation medium (LSM) (commercially available from Organon Teknika Corp., Durham, NC) and the tubes were spun at 1500 rpm for 30 minutes. The H9 cells, which separated into a distinct layer, were removed to another tube, mixed with 10 ml DMEM and spun at 2000 rpm for 5 minutes. The pellet was resuspended into 1 ml of 85% DMEM + 15% FBS, and then transferred to a 2 ml sartedt tube. The samples were also stored at -80°C.

The samples were titered using a microtiter plaque assay, as described in Example 8 for cell-free HIV and Example 9 for cell-associated HIV. The results appear in Table 13A (cell free) and 13B (cell associated), below.

TABLE 13A

Compound	Concentration (μ g/ml)	Log Titer pfu/ml	Log Reduction
QM	0	5.7	-
	10	3.9	1.8
	20	3.0	2.7
	40	0	>4.3
	80	0	>4.3
ICR-170	0	5.7	-
	10	5.1	0.6
	20	4.4	1.3
	40	3.4	2.3
	80	1.7	4.0

TABLE 13B

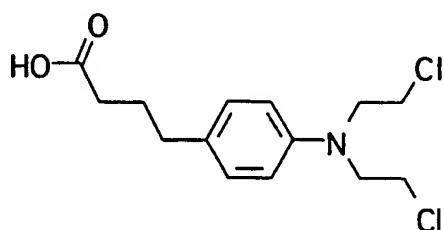
Compound	Concentration (μ g/ml)	Log Titer	Log Reduction
QM	0	5.5	-
	10	4.4	1.1
	20	3.6	1.9
	40	2.5	3.0
	80	2.4	3.1
ICR-170	0	5.7	-
	10	5.2	0.5
	20	4.5	1.2
	40	3.7	2.0
	80	3.7	2.0

5

EXAMPLE 10

The above examples have established that QM has exceptional pathogen inactivation activity. In choosing an agent to decontaminate blood products for clinical testing or transfusion, it is also important to consider the effects of the method and compound used on blood product function. This example explores 10 the short term effects of two compounds, one having a nucleic acid binding ligand and a mustard group, QM and chlorambucil on red blood cell function, as measured by potassium leakage and IgG binding to red blood cell surfaces. The structure of chlorambucil appears below.

15



20

This example additionally compares the R17 inactivation activity, in red blood cells, of a compound having both a nucleic acid binding ligand and a mustard group (QM), with a compound having only a mustard group, and no nucleic acid binding ligand (chlorambucil).

Whole blood (20 ml) was transferred to a 50 ml conical tube and spun down at 1600 rpm for 9 minutes at room temperature. Plasma was removed (9 ml). Next, 10.9 logs/ml stock of R17 phage was diluted 1:20 with Adsol (24.4 ml Adsol + 1.28 ml R17). The pelleted red blood cells were then resuspended to 5 30% Htc with 25.6 ml of the Adsol/R17 mixture. Aliquots (3 ml each) were transferred into 9 tubes on ice.

Each mustard was added to Adsol. Chlorambucil, commercially available from Aldrich Inc., Milwaukee, WI, (5.8 mg) was added to 1.93 ml Adsol plus 5.85 μ l 3M NaOH (undissolved material remained, and suspension was used in the 10 experiment by swirling before addition. QM (2.9 mg) was added to 0.967 ml Adsol (again, material remained in suspension). The mustards were immediately added to the blood, at volumes set forth in Table 14, below, and mixed by inversion.

TABLE 14

15

Sample	Contents	Volume Mustard
1	control	none
2	10 μ g/ml Chlorambucil	10 μ l
3	30 μ g/ml Chlorambucil	30 μ l
4	100 μ g/ml Chlorambucil	103 μ l
5	300 μ g/ml Chlorambucil	333 μ l
6	10 μ g/ml Quinacrine	10 μ l
7	30 μ g/ml Quinacrine	30 μ l
8	100 μ g/ml Quinacrine	103 μ l
9	300 μ g/ml Quinacrine	333 μ l

Extracellular potassium levels were measured approximately one hour after treatment using a Ciba Corning 614 K⁺/Na⁺ Analyzer (commercially available from Ciba Corning Diagnostics Corp., Medfield, Massachusetts). The 20 remaining samples were incubated overnight at 4°C. After incubation, 0.2 ml of each sample was removed for R17 assay and spun in a microfuge for 1 min.

Supernatant was then removed for phage assay.

Potassium levels on remaining samples were measured and the samples were stored at 4°C. Potassium measurements were repeated daily for one week 25 or until significant differences were observed. Extracellular potassium data appears in Table 15, and FIG. 6. IgG binding in the samples was measured using

Baxter Unival Anti-Human Globulin Anti-IgG for Direct Antiglobulin Test and Baxter Coombs control Cells for Quality Control of Anti-Human Globulin Test (both available from Baxter Healthcare Corporation, Deerfield, IL). The results of IgG Binding as measured by FACScan™ (Becton Dickinson, Mountain View, CA) appear in Table 16.

R17 was completely inactivated at all concentrations of QM (≥ 8.4 logs/ml). However, little or no inactivation (≤ 0.4 logs) was observed for Chlorambucil, up to a concentration of 300 μ g/ml.

10

TABLE 15

Sample	Extracellular Potassium (mM)						
	Day 0	Day 1	Day 2	Day 3	Day 4	Day 6	Day 7
1	0.70	1.59	2.51	3.16	3.71	4.63	5.01
2	0.76	1.59	2.45	3.18	3.72	4.57	4.94
3	0.69	1.56	2.40	3.16	3.72	4.69	5.03
4	0.72	1.74	2.43	3.18	3.76	4.73	5.12
5	0.72	1.71	2.58	3.31	3.92	4.89	5.36
6	0.73	1.65	2.76	3.64	4.26	5.30	5.69
7	0.76	1.94	3.08	4.00	4.63	5.76	6.15
8	0.78	2.48	4.05	5.23	6.06	7.50	8.02
9	0.82	3.61	5.59	7.37	8.32	>10	11.20

Packed red blood cells may be stored for up to 42 days, depending upon the storage conditions, according to standards set by the United States Food and Drug Administration. During this storage time, the level of extracellular potassium increases. For example, red blood cells stored for 35 days in citrate phosphate dextrose adenine-1 (CPDA-1) have displayed potassium levels as high as 78.5 mM/L, an increase of 73.4 mM/L over the potassium level at day one of storage. *Immunohematology Principles and Practice*, p. 29, Table 2-1, J.B. Lippincott Company, Philadelphia, PA (1993). Units bearing such levels of extracellular potassium are transfused without breaching acceptable standards of practice, and are thus considered by the FDA to retain the ability to function for the purpose they are prepared and used.

The literature further supports the assertion that increases in extracellular potassium, such as those in Table 15, are not an indication that the red cells have lost their ability to function for the purpose they were prepared.

Chlorambucil did not alter potassium leakage of red cells. QM showed 5 significant anti-viral activity and induced changes in potassium leakage that are within the range considered acceptable in stored red blood cells. The results indicate that the red blood cells retain the ability to function within the limits of the FDA standard.

10

TABLE 16

Sample	1	2	3	4	5	6	7	8	9
Median Fluorescence	3.43	3.31	3.37	3.31	3.62	5.00	7.77	15.8	48.7

Table 16 shows that Chlorambucil also did not alter IgG binding of red cells. There was an increase in the mean fluorescence from 3.43 for the control sample 15 to 48.7 for Sample 9, treated with 300 µg/ml quinacrine mustard. The assay used in this example was intended to measure fluorescence due to IgG binding. However, the assay also detects fluorescence from other sources. Quinacrine mustard contributes significantly to the level of fluorescence detected by the assay. The same samples tested in this example were also tested in a different 20 assay for IgG binding.

The following agglutination experiment was performed to determine whether or not the methods of the present invention stimulated IgG binding. IgG binding in the samples was measured using Baxter Unival Anti-Human Globulin Anti-IgG for Direct Antiglobulin Test and Baxter Coombs control Cells 25 for Quality Control of Anti-Human Globulin Test (both available from Baxter Healthcare Corporation, Deerfield, IL). The same samples 1-9 as described above were tested as follows: The packed red blood cell samples were diluted to 5% hematocrit. Approximately 50 µl of these diluted cells were then placed in a 1.7 ml tube, and 1 ml of saline was added to each. The samples were mixed and then 30 vortexed at 3000 rpm for 2 minutes. The supernatant was removed and the cells were washed three times with saline.

Next, 2 drops of Unival Anti-human Globulin was added to each sample and the samples were vortexed. Each sample was centrifuged for 20 seconds at 3430 rpm. None of the samples showed hemolysis after the centrifugation. The

cells were then gently resuspended and immediately examined for agglutination under microscope. All samples were negative for agglutination. Subsequently, one drop of Coombs positive control cells was added to the experimental samples. All samples then showed many medium and small agglutinates. The 5 results of this experiment indicate that the treatment of red blood cells by the methods of the present invention did not interfere with the agglutination assays. These assay results were duplicated in a blinded study, using material from the same samples, by an independent laboratory.

10 Results of the above described experiment clearly confirm that the methods of the present invention do not stimulate IgG binding. The increase in fluorescence in Table 16 was the result of fluorescence from QM rather than a significant increase in IgG binding.

EXAMPLE 11

15 Example 10 showed that QM was able to inactivate R17 in red blood cells under conditions where potassium leakage and surface IgG binding were negligible. This example is designed to further these observations by looking more extensively at red blood cell function after treatment with varying levels of QM. Specifically, this example looks at the effects of QM treatment on red blood 20 cell function after storage under conditions that closely mimic those in a blood bank.

25 A packed red blood cell unit, approximately 1 day old, was obtained from Sacramento Blood Center. The cells were resuspended and approximately 200 ml was transferred to a sterile container. R17 (0.2 ml) in LB was added and the sample was mixed. Next the unit was divided into 6-30 ml aliquots in sterile conical centrifuge tubes on ice. The remaining packed red blood cells were stored in the bag at 4°C.

QM (3.2 mg) was mixed with ice cold Adsol (1.6 ml) to make a 2.0 mg/ml suspension. Aliquots of the QM suspension were added to the cells as set forth 30 in Table 17. The samples were mixed thoroughly by gentle inversion and transferred to Fenwal transfer packs (Baxter/Fenwal, Ill) for storage at 4°C.

TABLE 17

Sample	Final Concentration of QM (µg/ml)	Volume of QM
1	0	0
2	2.5	37.5 µl
3	5	75 µl
4	10	0.15 ml
5	20	0.30 ml
6	40	0.60 ml

The following measurements of cell function were taken. 1) Potassium levels were determined daily for one week and weekly thereafter, using the Ciba Corning 614 K⁺/Na⁺ analyzer (commercially available from Ciba Corning, MA).
 5 2) Adenosine-5'-triphosphate (ATP) and 2,3-diphosphoglyceric acid (2,3-DPG) were measured the first day after treatment and weekly thereafter. ATP was measured using a Sigma ATP Kit, commercially available from Sigma, St. Louis MO, following Sigma Procedure No. 366-UV hereby incorporated by reference.
 10 2,3-DPG was measured using the 2,3-DPG Kit, commercially available from Sigma, St. Louis, MO. 3) IgG binding to the red blood cell surface was measured after day 1 and week 1, using the Baxter Unival Anti-Human Globulin Anti-IgG for Direct Antiglobulin Test and Baxter Coombs Control Cells for Quality Control of Anti-Human Globulin Test, commercially available from Baxter Healthcare, Inc., Deerfield, IL.

The results for R17 inactivation appear in FIG. 7. The results for red blood cell function appear in Tables 18A -18D.

TABLE 18A

QM Concentration	K ⁺ Day 1	K ⁺ Day 2	K ⁺ Day 7	K ⁺ Day 8	K ⁺ Day 9	K ⁺ Day 16
control	7.41	7.41	13.20	12.96	15.32	31.72
2.5 µg/ml	7.42	7.42	12.90	13.64	15.86	31.76
5 µg/ml	7.24	7.24	13.28	14.82	15.18	32.96
10 µg/ml	7.24	7.24	13.06	15.16	14.68	31.64
20 µg/ml	7.00	7.00	12.90	15.38	14.44	31.36
40 µg/ml	6.95	6.95	12.88	12.44	15.44	30.92

TABLE 18B

QM Concentration	ATP (mM)	ATP (mM)	ATP (mM)
	Day 1	Day 9	Day 16
control	0.77	0.80	0.75
2.5 µg/ml	0.78	0.80	0.74
5 µg/ml	0.78	0.81	0.73
10 µg/ml	0.76	0.81	0.73
20 µg/ml	0.78	0.80	0.73
40 µg/ml	0.76	0.80	0.72

TABLE 18C

5

QM Concentration	2,3-DPG	2,3-DPG	2,3-DPG
	Day 1	Day 9	Day 16
control	2.20	0.77	0.89
2.5 µg/ml	2.20	0.88	0.10
5 µg/ml	2.35	0.97	0.28
10 µg/ml	2.06	1.11	0.34
20 µg/ml	2.63	1.43	1.36
40 µg/ml	2.09	1.04	0.11

TABLE 18D

QM Concentration	mean FL	median FL	mean FL	median FL	mean FL	median FL
	Day 1	Day 1	Day 9	Day 9	Day 16	Day 16
control	4.41	4.1	4.41	4.1	4.41	4.1
2.5 µg/ml	4.77	4.45	4.77	4.45	4.77	4.45
5 µg/ml	4.79	4.45	4.79	4.45	4.79	4.45
10 µg/ml	4.96	4.7	4.96	4.7	4.96	4.7
20 µg/ml	5.73	5.19	5.73	5.19	5.73	5.19
40 µg/ml	6.31	6.04	6.31	6.04	6.31	6.04

10

Under conditions of effective R17 inactivation in packed red blood cells, there are no significant effects on potassium-leakage, ATP content, 2,3-DPG content, or IgG binding to RBCs.

EXAMPLE 12

This example evaluates QM to determine whether it is mutagenic in the Ames test, a well known assay for mutagenicity. While mustards are proving to be effective compounds for pathogen inactivation, they are also considered potential mutagens. This example shows that blood treated with QM does not exhibit significant mutagenic action, particularly after an incubation period. Thus, the compounds of the present invention have exceptional pathogen inactivation efficiency while displaying only minimal mutagenicity.

10 In this example QM was tested for its mutagenicity using an Ames assay. The mutagenicity was tested under four conditions: QM incubated overnight in water, QM added to red blood cells and immediately plated; QM added to red blood cells, incubated overnight at 4°C and then plated; and QM added to red blood cells, incubated 4 hours at 4°C, then mixed with Amberlite XAD-16™ and 15 incubated overnight before plating.

First, the solubility of QM in red blood cells was determined. 10 mg/ml QM was diluted 10-fold and 100-fold into red blood cells and the solubility was observed. To obtain a 1.0 mg/ml solution, 20 µl of the 10 mg/ml QM solution was combined with 180 µl of the 50% Htc red blood cells. This stock contained 20 definite particles. Next a 0.3 mg/ml concentration was tested by combining 20 µl of 3.0 mg/ml QM and 180 µl of 50% Htc red blood cells. There was evidence of precipitating out of solution with the 3 mg/ml stock. Finally, 20 µl of the 1.0 mg/ml stock was mixed with 180 µl of packed red blood cells. The 1.0 mg/ml stock appeared clear. The 3 mg/ml stock was chosen as the highest 25 concentration, thus 0.3 mg/ml in red blood cells and 30 µg/plate are the upper concentration limits in this experiment.

Preparation of these three test mixtures was as follows. A 10 mg/ml solution of QM in DMSO was diluted to 1.0 mg/ml (60 µl of QM solution added to 0.54 ml DMSO). A 50% Htc red blood cell solution was prepared by spinning 30 down 10 ml of a packed red cell unit at 1600 rpm for 9 minutes. Supernatant was removed and the cell pellet was resuspended in an equal volume of Adsol. Htc was then confirmed on a Model F800 Sysmex cell counter (Toa Medical Electronics, Kobe, Japan). Thirteen 0.9 ml aliquots of RBC solution were then placed in test tubes. Four different stock solutions of QM were prepared because 35 the compound may precipitate out of solution at concentrations as low as 3 mg/ml. Stock solutions at varying concentrations were prepared by making the

following dilutions of a 1.0 mg/ml solution: 150 μ l of a 1.0 mg/ml QM solution + 350 μ l DMSO to produce a 0.3 mg/ml solution; 40 μ l of a 1.0 mg/ml QM solution + 360 μ l DMSO to produce a 0.1 mg/ml solution; 15 μ l of a 1.0 mg/ml QM solution + 485 μ l DMSO to produce a 0.03 mg/ml solution. To the first tube, 5 100 μ l DMSO was added and the tube was placed on a 4°C shaker (25 rpm, Orbital Shaker, commercially available from VWR Scientific, Greenbelt, MO) for overnight incubation. Tubes 2 - 5 were shaken overnight as well, then 100 μ l aliquots of each QM solution was diluted into the tubes just before addition to the Ames strains. To tubes 6 - 9 was added 100 μ l of each QM solution. The tubes 10 were then incubated overnight at 4°C on the shaker. Finally, 100 μ l of each QM solution was also added to tubes 10 - 13, which were then incubated on the shaker for 4 hours. Subsequently, 0.1 g of a polymeric adsorbent material, 15 Amberlite XAD 16TM (commercially available from Sigma, Saint Louis, MO), was added to each of tubes 10 - 13 and the incubation was continued overnight. The final contents of each tube, and the stock QM solutions used, are listed in Table 19, below.

TABLE 19

SAMPLE NUMBER	CONTENTS	QM STOCK SOLUTION
1	RBC + DMSO	none
2	RBC + 0.003 mg/ml QM	0.03 mg/ml
3	RBC + 0.01 mg/ml QM	0.1 mg/ml
4	RBC + 0.03 mg/ml QM	0.3 mg/ml
5	RBC + 0.1 mg/ml QM	1 mg/ml
6	RBC + 0.003 mg/ml QM	0.03 mg/ml
7	RBC + 0.01 mg/ml QM	0.1 mg/ml
8	RBC + 0.03 mg/ml QM	0.3 mg/ml
9	RBC + 0.1 mg/ml QM	1 mg/ml
10	RBC + 0.003 mg/ml QM	0.03 mg/ml
11	RBC + 0.01 mg/ml QM	0.1 mg/ml
12	RBC + 0.03 mg/ml QM	0.3 mg/ml
13	RBC + 0.1 mg/ml QM	1 mg/ml

20 In a separate experiment, samples of QM in water were prepared as follows. Sample tubes were labeled and .5 ml phosphate buffer was added to each one. Then various dilutions of a stock solution of QM (1 mg/ml) were added to five

of the tubes. For 100 µg/plate - 1.4 ml stock solution; for 30 µg/plate - .42 ml stock + .98 ml H₂O; for 10 µg/plate - .14 ml stock + 1.26 ml H₂O; for 3 µg/plate - .042 ml stock + 1.358 ml H₂O; for 1 µg/plate - .014 ml stock + 1.386 ml H₂O. A control was also prepared using only H₂O.

5 The procedures used for the Salmonella mutagenicity test as described in detail by Maron and Ames were followed exactly. Maron, D.M. and B.N. Ames, Mutation Research 113: 173 (1983). A brief description for each procedure is given here. The tester strains TA97a, TA98, TA100, TA102, TA1537 and TA1538 were obtained from Dr. Ames. TA97a, TA98, TA1537 and TA1538 are frame shift 10 tester strains. TA100 and TA102 are base-substitution tester strains. Upon receipt each strain was cultured under a variety of conditions to confirm the genotypes specific to the strains.

15 The standard Salmonella tester strains used in this study require histidine for growth since each tester strain contains a different type of mutation in the histidine operon. In addition to the histidine mutation, these tester strains contain other mutations, described below, that greatly increase their ability to detect mutagen.

20 *Histidine Dependence:* The requirement for histidine was tested by streaking each strain first on a minimal glucose plate supplemented only with biotin and then on a minimal glucose plate supplemented with biotin and histidine. All strains grew only on the histidine/biotin supplemented plates, confirming a histidine requirement.

25 *rfa Mutation:* A mutation which causes partial loss of the lipopolysaccharide barrier that coats the surface of the bacteria thus increasing permeability to large molecules was confirmed by exposing a streaked nutrient agar plate coated with the tester strain to crystal violet. First 100 µL of each culture was added to 2 mL of molten minimal top agar and poured onto a nutrient agar plate. Then a sterile filter paper disc saturated with crystal violet was placed at the center of each plate. After 16 hours of incubation at 37°C the 30 plates were scored and a clear zone of no bacterial growth was found around the disc, confirming the rfa mutation.

35 *uvrB Mutation:* Three strains used in this study contain a deficient UV repair system (TA97a, TA98, TA100, TA1537 and TA1538). This trait was tested for by streaking the strains on a nutrient agar plate, covering half of the plate, and irradiating the exposed side of the plate with germicidal lamps. After

incubation growth was only seen on the side of the plate shielded from UV irradiation.

5 *R-factor*: The tester strains (TA97a, TA98, TA100, and TA102) contain the pKM101 plasmid that increases their sensitivity to mutagens. The plasmid also confers resistance to ampicillin to the bacteria. This was confirmed by growing the strains in the presence of ampicillin.

10 *pAQ1*: Strain TA102 also contains the pAQ1 plasmid that further enhances its sensitivity to mutagens. This plasmid also codes for tetracycline resistance. To test for the presence of this plasmid TA102 was streaked on a minimal glucose plate containing histidine, biotin, and tetracycline. The plate was incubated for 16 hours at 37°C. The strain showed normal growth indicating the presence of the pAQ1 plasmid.

15 The same cultures used for the genotype testing were again cultured and aliquots were frozen under controlled conditions. The cultures were again tested for genotype to confirm the fidelity of the genotype upon manipulation in preparing the frozen permanents.

20 The first tests done with the strains were to determine the range of spontaneous reversion for each of the strains. With each mutagenicity experiment the spontaneous reversion of the tester strains to histidine independence was measured and expressed as the number of spontaneous revertants per plate. This served as the background controls. A positive mutagenesis control was included for each tester strain by using a diagnostic mutagen suitable for that strain (2-aminofluorene at 5 mg/plate for TA98; sodium azide at 1.5 mg/plate for TA100; 9-aminoacridine for TA 1537).

25 For all experiments, the pre-incubation procedure was used. In this procedure one vial of each tester strain was thawed and tubes were prepared for each strain, containing 20 µL of the culture and 6 mL of Oxoid Nutrient Broth #2. This solution was allowed to shake for 10 hours at 37°C. In the pre-incubation procedure, for each tester strain used to evaluate the test solution, 0.1 mL of the overnight culture was added to each of 13 sterile test tubes. To each of the tubes, 0.1 mL of the test solution from tubes 1 - 13 was added. This was also performed on the samples containing QM in water only. Then 0.5 mL of 0.2 M sodium phosphate buffer, pH 7.4 was added. The 0.7 mL mixture was vortexed and then pre-incubated while shaking for 20 minutes at 37°C. After shaking, 2 mL of molten top agar supplemented with histidine and biotin were added to the 0.7 mL mixture and immediately poured onto a minimal glucose agar plate

(volume of base agar was 20 mL). The top agar was allowed 30 minutes to solidify and then the plates were inverted and incubated for 44 hours at 37°C. After incubation, the number of revertant colonies on each plate was counted.

5 The results appear in FIG. 8. Although the QM registered a positive response in the Ames test without incubation in red blood cells and in water, an overnight incubation in red blood cells significantly reduced the level of revertants, as did an incubation with adsorbent material. A parallel experiment was performed using an activated charcoal adsorbent material, Hemosorba (commercially available from Asahi Medical Corp., Tokyo, Japan.) The results, 10 which are not shown, were similar to the results using Amberlite XAD 16™.

EXAMPLE 13

As discussed above, a method of decontaminating clinical samples would be most useful if while it decontaminated samples, it did not significantly effect 15 the results of the clinical tests themselves. This example compares results of a common blood chemistry panel for samples treated by methods of the present invention to untreated samples.

Solutions of QM and ICR-170 (2 mg/ml) were prepared in saline. The QM was almost completely dissolved, and ICR-170 remained a suspension. Next, 20 human whole blood was drawn and 10 ml aliquots were placed in eight tubes. QM or ICR-170 was added to six of the tubes in aliquots of 100, 200, or 400 µl, to reach final concentrations of the compounds of either 20, 40, or 80 µg/ml. Saline was added to the remaining two tubes, in aliquots of 100 or 400 µl, to prepare control samples. The samples were then allowed to clot for 30 minutes, followed 25 by 20 minutes on a centrifuge at 1000 rpm. The separated serum was then transferred to labeled plastic tubes and tested in a panel of 24 common clinical chemistry tests.

The results appear below, in Table 20. Neither QM nor ICR-170 had a significant effect on the results of any of the panel of 24 clinical chemistry tests. 30 Lactate dehydrogenase and GGT exhibited a small drop in the sample containing the highest concentration of ICR-170. Clearly, the methods of the present invention do not interfere significantly with clinical testing of blood samples.

TABLE 20

TEST	100 µl saline	400 µl saline	20 µg/ml QM	40 µg/ml QM	60 µg/ml QM	20 µg/ml ICR170	40 µg/ml ICR170	60 µg/ml ICR170
Glucose	95	92	93	93	90	94	92	90
BUN	17	17	17	17	17	17	17	17
Creatinine	1.1	1.1	1.1	1.2	1.1	1	1.1	1.1
Bun/Creat. ratio	15	15	15	14	15	17	15	15
Sodium	143	143	138	144	146	143	144	144
Potassium	4	3.9	4	3.9	3.9	4	4.1	4.1
Chloride	104	106	104	106	107	103	104	103
Magnesium	1.7	1.6	1.6	1.6	1.6	1.7	1.6	1.5
Calcium	9.3	8.8	9.3	8.8	9	9.4	9.2	9.1
Phosphorous inorganic	4.3	3.9	4.1	4.1	4.1	4.3	4.3	4.1
protein, total	7.3	7.1	7.4	7.3	7.1	7.3	7.3	7.1
albumin	4.6	4.5	4.6	4.6	4.4	4.6	4.6	4.4
globulin, total	2.7	2.6	2.8	2.7	2.7	2.7	2.7	2.7
A/G ratio	1.7	1.7	1.6	1.7	1.6	1.7	1.7	1.6
bilirubin	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6
alk	55	50	48	50	51	54	51	47
LDH	136	134	121	125	138	129	138	151
GGT	23	21	22	22	22	23	20	16
AST	17	16	18	16	16	16	17	16
ALT	20	19	17	19	18	19	19	19
Uric Acid	5.6	5.3	5.5	5.4	5.4	5.7	5.6	5.5
Iron	119	107	117	113	115	119	122	120
Triglyceride	164	152	159	158	158	161	150	156
Cholesterol	234	221	227	226	223	231	231	223

EXAMPLE 14

5 This example describes the inactivation of bacterial pathogens of biological compositions using methods of the present invention. The following experiment was performed to support that the methods of the present invention can be used to inactivate bacterial pathogens. In this example, the decontamination methods of the present invention were applied to inactivate Staphylococcus epidermidis.

10 An overnight culture of the organism was made by inoculating 3 ml of LB broth from a motility stab. This was maintained at 35°C and 1.0 ml of it was used to inoculate 9 ml of LB broth in a 15 ml conical tube. A sample (1 ml) was taken for an OD₆₀₀ reading. To a tube, 5 ml of LB broth was added. Then 50 µl of 10⁸ cfu/ml S. epidermidis was added. Aliquots of the sample (1 ml each) were placed in 5 tubes. These were treated with either 0, 3, 10, 30, or 100 µg/ml of QM. A 2 15 mg/ml stock of QM in ddH₂O was added in the following amounts to produce the desired concentrations: 0µl, 1.5µl, 5 µl, 15 µl, and 50 µl. The samples were incubated on ice for three hours.

20 After incubation, bacteria was quantified by plating 0.1 ml of serial 10-fold dilutions in LB broth onto 100 mm petri dishes containing agar. After 24 hr. incubation at 35°C, colonies were counted and bacterial concentration was calculated on a per ml basis. The results, which appear in FIG. 9, show that QM at >10 µg/ml inactivates S. epidermidis to the level of detection of this assay.

EXAMPLE 15

25 If a decontaminated blood product is to have value as an *in vivo* therapy, the blood product must retain some efficacy after the decontamination process. One way to confirm the efficacy of a particular sample of red blood cells is to ensure that they are not cleared by the recipient's body significantly sooner than normal red blood cells when transfused into a mammal. In this example, packed 30 red blood cells are treated with compounds having a nucleic acid binding ligand and a mustard group, according to the methods of the present invention, transfused into mice, and tracked for post transfusion survival.

35 Blood was drawn from 8 Balb/c mice using an anticoagulant (containing citrate, ethylene diamine tetraacetic acid, prostaglandin E1 and theophyllin) for a total of 12 ml (8 ml whole blood and 4 ml anticoagulant). An equivalent volume of Adsol was added and the sample was centrifuged at 2000 rpm for 5 minutes.

The supernatant was removed and saved as "washed solution". The red blood cell pellet was resuspended in Adsol to make a 50% Htc solution. Three 1.5 ml aliquots were transferred to 14 ml round bottom polypropylene tubes.

5 Next, 1 mg/ml solutions of two compounds, QM and ICR-170, were prepared in saline. The compounds were added to the three polypropylene tubes as follows: tube 1 received no treatment (120 μ l of saline was added); tube 2 received 120 μ l of the 1 mg/ml solution of QM for a final concentration of 80 μ g/ml; tube 3 120 μ l of the 1 mg/ml solution of ICR-170, for a final concentration of 80 μ g/ml.

10 The samples were then incubated for 2 hours at 4° C. Cells were washed three times, each time by adding 6 ml Adsol to each tube and spinning the samples at 1800 rpm for 5 minutes. After the final wash, the pellet was resuspended in Adsol buffer to a concentration of 4×10^6 cells/ μ l. 50 μ l of each sample was removed for an unstained control.

15 The remaining cells were then stained with PKH26 dye. From a 1mM PKH26 stock, 120 μ l was removed and diluted with 8 ml diluent A to create a working solution of 15.7 μ M PKH26. This solution was stored in the dark until use. To each 2 ml of cells, 2 ml of PKH26 dye was added. The samples were mixed gently and incubated for 5 minutes at room temperature in the dark. The 20 cells were remixed after 2.5 minutes. After another 5 minute incubation, 2 volumes of the reserved "washed solution" was added to stop the staining reaction. The cells were centrifuged at 1800 rpm for 5 minutes to pellet and the supernatant was removed. The cells were then washed 3 times with Adsol buffer, as before. After the final wash, the sample volume was restored to 1 ml 25 with Adsol. An aliquot of each sample was removed at this point for a positive stained control sample and counted on the Sysmex machine.

30 Swiss Webster mice were transfused with 0.2 ml of the labeled cells from each of samples 1-3 via tail vein injection. The mice were then weighed to calculate the blood volume. Blood volume is calculated as the animal weight in gm \times 0.06. Then, blood was drawn from the mice by retroorbital venipuncture using heparin-EDTA coated capillary tubes at 1 hour, 24 hours, 2 more times during the first week, and one time weekly for 3 weeks. The eye bleeding samples were drained into isotonic solution before analysis.

35 Samples were analyzed on a FACScan™ at the FL2 (red fluorescent channel) with gating on the red cell population using forward and side scatter

linear mode gates. The proportion of labeled cells in 100,000 total red cell gated events was determined.

TABLE 21

SAMPLE	RECOVERY	LOSS PER DAY
Untreated	90.3 ± 3.2	2.66%
QM	80.8 ± 4.7	2.77%
ICR-170	86.0 ± 6.6	2.71%

5

The results appear in Table 21. According to the results, regardless of treatment, treated cells survived *in vivo* as well as untreated control cells.

Some hemolysis of RBC was detected after labeling with PKH26. Thus, recoveries may be effected by the labeling technique. An alternative labeling 10 technique was also used, as described. An activated biotin ester was injected via the tail vein of the Balb/c mice in order to label the red cells *in vivo* (each mouse received either a "low dose treatment" - 0.1 mg injection on two successive days, or a "high dose treatment" - 0.3 mg of biotin on three successive days). After treatment, low and high dose treatment cells with fluorescence 15 tagged streptavidin were clearly distinguishable as detected by FACScan analysis. Low and high dose treatment cells were independently treated with 80 µg/ml of QM or ICR-170 as described above. After treatment, cells were washed, mixed with untreated cells that had been differentially labeled. Then three mice were transfused: one received untreated low and untreated high dose RBC, one 20 received QM treated low and untreated high dose RBC, and one received ICR-170 treated low and untreated high RBC. Bleeding was performed as described above. The results appear in Table 22, below. Clearly, recovery of the treated cells is very similar to the untreated cells.

25

TABLE 22

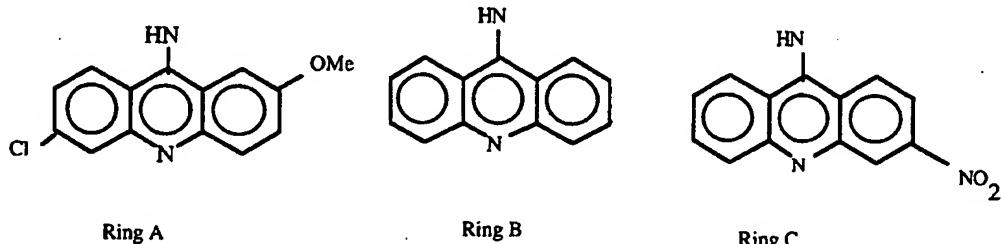
	% RECOVERY, LOW	% RECOVERY, HIGH
untreated	96.2	93.5
QM	94.6	91.2
ICR-170	93.1	90.4

EXAMPLE 16

This example sets forth the results of several screens for viral inactivation performed on compounds of the present invention which have acridine groups as their nucleic acid binding ligands. Descriptions of how these screens were 5 performed are found in the above examples discussing R-17, cell free HIV and cell associated HIV. Table 23, below, shows results in terms of the anti-viral activity of nucleic acid specific alkylating agents comprising: acridine ring - linking chain - N(CH₂CH₂Cl)₂. Activity is set forth by + for lowest, up to ++++ for complete inactivation (to the level of detection used).

10

TABLE 23



Cmpd	Ring	linker	R17 ^a	CA-HIV ^b	CF-HIV ^b
2	A	(CH ₂) ₂	+		
3	A	(CH ₂) ₃	++	+	+
4	B	(CH ₂) ₃		+	+
5	A	(CH ₂) ₄	++++	++++	+++
1	A	CH(CH ₃)(CH ₂) ₃	++++	+++	+++
6	A	(CH ₂) ₅	++++	++++	+++
7	B	(CH ₂) ₅		++++	++++
8	C	(CH ₂) ₅		+	++
9	A	(CH ₂) ₆	++++	+++	++
10	B	(CH ₂) ₆		++++	+++
11	A	CH ₂ CONH(CH ₂) ₃	++++	+++	++
12	A	CH(CH ₃)CONH(CH ₂) ₂	++++	+++	+
13	A	CH(CH ₃)CONH(CH ₂) ₃	++++	+++	+

^a + + + + shows ≥ 6 log reduction at 5 μ M compound; ^b + + + + shows ≥ 4 log reduction at 20 μ M.

It is clear from these data that the acridine based compounds all have inactivation activity against all pathogens tested, including cell free, cell associated HIV and R-17.

5 Nucleic acid specific alkylating agents tested here have been prepared by methods described in [Peck, R. M., Preston, R. K., Creech, H. J., *J. Amer Chem. Soc* (1959) 81, 3984; Preston, R. K., Peck, R. M., Breuniger, E., R., Miller, A. J., Creech, H. J., *J. Med. Chem.* (1964) 7, 471]

EXAMPLE 17

10 This example describes the synthesis of a compound of the present invention, 5-[N,N-bis(2-chloroethyl)amino]methyl-8-methoxypsoralen hydrochloride (referred to throughout the text as "Compound 1").

Step 1: The synthesis of 5-Bromomethyl-8-methoxypsoralen.

15 To a solution of 2.69 g (12.3 mmol) of 8-methoxypsoralen (commercially available from Aldrich, Milwaukee, WI) in 135 mL of glacial acetic acid was added 11 mL of bromomethyl methyl ether. The solution was swirled, then left for three days at room temperature during which a white solid precipitated. The mixture was cooled in an ice bath and filtered. To the filtrate was added an
20 additional 2.75 g (12.7 mmol) of 8-methoxypsoralen and 5 mL of BrCH₂OCH₃. After again sitting for three days, product isolation was repeated. The filter cakes were washed with cold glacial acetic acid, air dried and finally vacuum dried to give a total yield of 6.3 g (81%) of 5-bromomethyl-8-methoxypsoralen as a pale yellow solid. NMR (CDCl₃): 4.33 (s, 3H), 4.88 (s, 2H), 6.52 (d, J = 10 Hz, 1H), 6.95
25 (d, J = 2 Hz, 1H), 7.77 (d, J = 2 Hz, 1H), 8.11 (d, J = 10 Hz, 1H).

Step 2: The synthesis of 5-[N,N-Bis-(2-hydroxyethyl)amino]methyl-8-methoxypsoralen:

30 5-Bromomethyl-8-methoxypsoralen (0.50g, 1.6 mmol, from Step 1, above) and diethanolamine (2.56 mL, 27 mmol) were combined in 23 mL of absolute ethanol and refluxed for 10 hours. The solution was concentrated, then CHCl₃ (65 mL) was added to the residue. The organic layer was washed with 30, 30 and 10 mL of water sequentially and the combined aqueous solutions were back extracted with CHCl₃. The combined organic solutions were then extracted three
35 times, each time with 7 mL of 1.2 N HCl. The combined acid solution was taken to pH 5-6 with 10% aqueous NaOH and the resultant turbid solution was washed

4 times, each time with 20 mL of CHCl₃. This last organic solution was rinsed with 2 x 20 mL of brine, dried (Na₂SO₄) and concentrated to give 0.43 g (79%) of the aminediol, 5-[N,N-bis-(2-hydroxyethyl)amino]methyl-8-methoxypsoralen, melting point 121-122 °C; NMR (CDCl₃): 2.71 (t, J = 5 Hz, 4H), 3.61 (t, J = 5 Hz, 4 H), 4.09 (s, 2H), 4.29 (s, 3H), 6.41 (d, J = 10 Hz, 1H), 7.00 (d, J = 2 Hz, 1H), 7.70 (d, J = 2 Hz, 1H), 8.38 (d, J = 10 Hz, 1H).

Step 3: 5-[N,N-Bis(2-chloroethyl)amino]methyl-8-methoxypsoralen hydrochloride.

10 5-[N,N-Bis(2-hydroxyethyl)amino]methyl-8-methoxypsoralen (0.030 g, 0.090 mmol) was dissolved in 1 ml thionyl chloride. It was covered with a serum cap with a small needle vent and allowed to stir for 3 days. The reaction mixture was stripped and the crude solid was recrystallized in isopropanol to give 5-[N,N-bis(2-chloroethyl)amino]methyl-8-methoxypsoralen hydrochloride (0.012 g, 15 32.4%) as an off-white solid, mp 158-162 °C. ¹HNMR (CD₃OD): 3.40 (t, J = 6Hz, 4H), 3.86 (t, J = 6Hz, 4H), 4.33 (s, 3H), 4.70 (s, 2H), 6.52 (d, J = 10Hz, 1H), 7.28 (d, J = 2Hz, 1H), 8.03 (d, J = 2Hz, 1H), 8.48 (d, J = 10Hz, 1H). The chemical shift appeared to be sensitive to trace acid present.

20 A portion of the above salt was partitioned between methylene chloride and aqueous NaHCO₃. The organic layer was again washed with aqueous bicarbonate, dried with brine, then dried with anhydrous Na₂SO₄ and evaporated to give the neutral amine; mass spectrum (EI, m/e): 371 (3), 369 (4), 230 (16), 229 (100), 214 (5), 201 (5), 186 (10) (obtained on a Shimadzu QP5000 GC/MS, with Rtx-5, 15m column, commercially available from Shimadzu 25 Corporation, Kyoto, Japan).

EXAMPLE 18

30 This example describes a contemplated embodiment wherein red blood cells are treated by a method of the present invention. The standard blood product separation approach used presently in blood banks is as follows: three bags are integrated by flexible tubing to create a blood transfer set (e.g., commercially available from Baxter, Deerfield, Ill.). After blood is drawn into the first bag, the entire set is processed by centrifugation (e.g., SorvallTM swing 35 bucket centrifuge, Dupont), resulting in packed red cells and platelet rich plasma in the first bag. The plasma is expressed off of the first bag (e.g., using a

Fenwall™ device for plasma expression), through the tubing and into the second bag. The first bag, containing packed red cells, is then detached.

In one embodiment of the decontamination approach of the present invention applied specifically to red blood cells, a compound having a nucleic acid binding ligand and a mustard group is introduced to the red blood cells (e.g. 5 the compound may be present in the first bag before blood is drawn, or transferred to the first bag after centrifugation), mixed and incubated. After 10 incubation, the compound may be removed using an adsorbent material (e.g., a commercially available material, such as activated charcoal or an Amberlite resin). The adsorbent may be introduced directly into the bag containing the red 15 blood cells, or the red blood cells may be passed through a removal device which contains the adsorbent. Alternatively, the compound may be removed by a cell washing process. The incubation, removal, and any subsequent storage, may take place in a commercially available storage bag.

From the above, it should be evident that the present invention provides 15 methods of decontamination of blood preparations intended for storage and *in vivo* use.

EXAMPLE 19

20

This example describes the synthesis of 8-[3-(Bis-2-chloroethyl)amino]propyloxypsoralen (compound 5)

STEP 1: 8-(3-Bromopropoxy) psoralen

1,3-Dibromopropane (7 mL, 68.9 mmol) was added to a slurry of 8-25 hydroxypsoralen (1.00 g, 4.95 mmol) in acetone (100 mL). After the addition of powdered K₂CO₃ (3.0 g, 21.7 mmol, 325 mesh), the mixture was refluxed for 6 h, allowed to cool to room temperature, and filtered to remove K₂CO₃. The salt 30 was rinsed with CH₂Cl₂ and the combined filtrates were concentrated. To remove residual dibromopropane the precipitate was triturated with hexane, then recrystallized in methylcyclohexane to give 8-(3-bromopropoxy)psoralen (1.19 g, 56.9% yield), as a beige solid. ¹H NMR (CDCl₃): δ 2.41 (quintet, J = 5.9 Hz, 2H), 3.78 (t, J = 6.5 Hz, 2H), 4.64 (t, J = 5.8 Hz, 2H), 6.38 (d, J = 9.6 Hz, 1H), 6.82 (d, J = 2.2 Hz, 1H), 7.71 (d, J = 2.2 Hz, 1H), 7.78 (d, J = 9.6 Hz, 1H).

STEP 2: 8-[3-(Bis-2-hydroxyethyl)amino]propyloxypsoralen

35 8-(3-Bromopropoxy)psoralen (0.500 g, 1.55 mmol) and diethanolamine (1.5 mL, 15.6 mmol) were refluxed in ethanol (20 mL) overnight. After rotovapping

off the solvent, the resulting syrup was dissolved in CH_2Cl_2 , washed with water several times, then brine, dried with anhydrous Na_2SO_4 and stripped of solvent to give 8-[3-(bis-2-hydroxyethyl)amino]propyloxypsoralen (0.499 g, 92.9% yield), as a brown syrup. ^1H NMR (CDCl_3): δ 2.04 (quintet, $J = 6.2$ Hz, 2H), 2.72 (t, $J = 5.2$ Hz, 4H), 2.91 (t, $J = 6.6$ Hz, 2H), 3.70 (t, $J = 5.3$ Hz, 4H), 4.54 (t, $J = 5.7$ Hz, 2H), 6.38 (d, $J = 9.6$ Hz, 1H), 6.82 (d, $J = 2.1$ Hz, 1H), 7.39 (s, 1H), 7.70 (d, $J = 2.1$ Hz, 1H), 7.79 (d, $J = 9.5$ Hz, 1H).

STEP 3: 8-[3-(Bis-2-chloroethyl)amino]propyloxypsoralen, (compound 5)

10 Thionyl chloride (0.030 mL, 0.41 mmol) was added dropwise to an ice bath chilled solution of 8-[3-(Bis-2-hydroxyethyl)amino]propyloxypsoralen (20.0 mg, 0.0575 mmol) and pyridine (0.016 mL, 0.21 mmol) in benzene (2 mL) and CH_2Cl_2 (2 mL). The reaction mix was covered with a serum cap and allowed to stir overnight at room temperature, then stripped under reduced vacuum and 15 partitioned between CH_2Cl_2 and 10 % NaHCO_3 . The organic layer was washed several times with aqueous NaHCO_3 , then brine. After drying with anhydrous Na_2SO_4 , solvent was rotovapped off to give 8-[3-(bis-2-chloroethyl)amino]propyloxypsoralen (14.0 mg, 57.8 % yield), as a yellow solid. ^1H NMR (CDCl_3): δ 1.97 (app quintet $J = 6.3$ Hz, 2H), 2.91 (app t, $J = 7.5$ Hz, 6H), 20 3.55 (t, $J = 7.0$ Hz, 4H), 4.58 (t, $J = 5.9$ Hz, 2H), 6.37 (d, $J = 9.5$ Hz, 1H), 6.82 (d, $J = 2.1$ Hz, 1H), 7.37 (s, 1H), 7.70 (d, $J = 2.1$ Hz, 1H), 7.77 (d, $J = 9.5$ Hz, 1H).

8-[5-(Bis-2-chloroethyl)amino]pentyloxypsoralen (compound 6)

25 In the same manner as the foregoing, but using 1,5-dibromopentane in Step 1 in place of 1,3-dibromopropane, compound 6 is produced.

EXAMPLE 20

This example describes the synthesis of 5-[3-(Bis-2-chloroethyl)aminopropyl]methoxy]methyl-8-methoxypsoralen, (Compound 4)

STEP 1: 5-[(3-Hydroxy)-propyloxy]methyl-8-methoxypsoralen

30 5-Bromomethyl-8-methoxypsoralen[®] (0.300 g, 0.970 mmol) and 1,3-propanediol (4 mL, 55.3 mmol) were refluxed in acetone (30 mL) for 3 days. The solvent was removed under reduced pressure and the residue was dissolved in CH_2Cl_2 and washed several times with water to remove excess diol. After

preliminary drying with brine then anhydrous Na₂SO₄, the solvent was rotovapped off to give 5-[(3-hydroxy)-propyloxy]methyl-8-methoxypsoralen (0.194 g, 65.8 % yield), as a yellow solid. ¹H NMR (CDCl₃): d 1.86 (quintet, J = 5.8 Hz, 2H), 3.68 (t, J = 5.9 Hz, 2H), 3.75 (t, J = 5.8 Hz, 2H), 4.30 (s, 3H), 4.89 (s, 2H), 6.43 (d, J = 9.9 Hz, 1H), 6.95 (d, J = 2.2 Hz, 1H), 7.71 (d, J = 2.2 Hz, 1H), 8.14 (d, J = 9.9 Hz, 1H).

5 STEP 2: 5-[(3-Methanesulfonyl)propyloxy]methyl-8-methoxypsoralen
A solution of 5-(1-hydroxy-propoxy)methyl-8-methoxy psoralen (0.194 g, 0.637 mmol) in CH₂Cl₂ (4 mL) was chilled with an ice/water bath.
10 Triethylamine (0.28 mL, 2.04 mmol) then methanesulfonyl chloride (0.15 mL, 1.91 mmol) were added dropwise. The solution was allowed to warm to room temperature and stirred for 4-6 h. The reaction mixture was partitioned between CH₂Cl₂ and water and the organic layer was washed several times with water, then brine, and dried with anhydrous Na₂SO₄ and concentrated to give crude 5-
15 [(3-methanesulfonyl)propyloxy]methyl-8-methoxypsoralen (0.266 g, 109 % yield) which was used directly for the next step.

20 STEP 3: 5-[3-(Bis-2-hydroxyethyl)aminopropyloxy]methyl-8-methoxypsoralen
A solution of 5-[(3-methanesulfonyl)propyloxy]methyl-8-methoxypsoralen (0.266 g, 0.695 mmol) and diethanolamine (0.500 g, 4.76 mmol) were refluxed in acetonitrile (6 mL) overnight. After rotovapping off the solvent, the resulting syrup was dissolved in CH₂Cl₂, washed with water several times, then brine, dried with anhydrous Na₂SO₄ and stripped to give 5-[3-(Bis-2-
25 hydroxyethyl)aminopropyloxy]methyl-8-methoxypsoralen (0.217g, 82.8 % yield), as a yellow solid. ¹H NMR (CDCl₃): d 1.76 (quintet, J = 6.1 Hz, 2H), 2.60-2.67 (m, 6H), 3.55-3.63 (m, 6H), 4.29 (s, 3H), 4.88 (s, 2H), 6.43 (d, J = 9.9 Hz, 1H), 6.98 (d, J = 2.1 Hz, 1H), 7.71 (d, J = 2.2 Hz, 1H), 8.15 (d, J = 9.9 Hz, 1H).

30 STEP 4: 5-[3-(Bis-2-chloroethyl)aminopropyloxy]methyl-8-methoxypsoralen, (compound 4)

Thionyl chloride (0.040 mL, 0.55 mmol) was added dropwise to an ice bath chilled solution of 5-[3-(bis-2-hydroxyethyl)aminopropyloxy]methyl-8-
35 methoxypsoralen (10.0 mg, 0.0255 mmol) and pyridine (0.040 mL, 0.49 mmol) in CH₂Cl₂ (2 mL). The reaction mix was covered with a serum cap and allowed to stir 5 h at room temperature under nitrogen, then stripped under reduced

vacuum and partitioned between CH_2Cl_2 and 10 % NaHCO_3 . The organic layer was washed several times with aqueous NaHCO_3 , then brine. After drying with anhydrous Na_2SO_4 , solvent was rotovapped off to give 5-[3-(bis-2-chloroethyl)aminopropoxy]methyl-8-methoxysoralen (0.0075 mg, 63.0 % yield), as a syrup. ^1H NMR (CDCl_3): δ 1.71 (t, J = 6.1 Hz, 2H), 2.60 (t, J = 6.4 Hz, 2H), 2.82 (t, J = 6.6 Hz, 4H), 3.3-3.7 (m, 6H), 4.29 (s, 3H), 4.86 (s, 2H), 6.43 (d, J = 9.9 Hz, 1H), 6.96 (s, 1H), 7.71 (s, 1H), 8.15 (d, J = 9.9 Hz, 1H).

EXAMPLE 21

10

This example describes the synthesis of 5-[3-(Bis-2-chloroethyl)aminopropoxy]methyl-8-methoxysoralen, (compound 16)

15 STEP 1: 5'-[4-(Bis-2-hydroxyethyl)amino-1-butylaminomethyl]-4,4',8-trimethylpsoralen

A solution of 5'-bromomethyl-4,4',8-trimethylpsoralen (US Patent 4,294,822, 73.0 mg, 0.227 mmol), and N,N-bis(2-hydroxyethyl)-1,4-butanediamine (400 mg, 2.27 mmol) were stirred in acetonitrile (100 mL) for 4 h. After rotovapping off the solvent, the resulting syrup was dissolved in CH_2Cl_2 ,

20 washed with 0.3M HCl several times, then chilled in an ice/water bath and made basic with powdered K_2CO_3 . The product was extracted from the aqueous layer with several portions of CH_2Cl_2 . The organic solution was rinsed with brine then dried with anhydrous Na_2SO_4 and stripped to give a brown syrup. The crude product was chromatographed by TLC (silica gel, 1/9 MeOH/ CHCl_3)

25 dissolved in CH_2Cl_2 to give 5'-[4-(bis-2-hydroxyethyl)amino-1-butylaminomethyl]-4,4',8-trimethylpsoralen, as a yellow syrup (26.9 mg, 29 % yield). ^1H NMR (CDCl_3): δ 1.59 (s, 3H), 2.49-2.76 (m, 14H), 3.62 (t, J = 5.3 Hz, 4H), 3.97 (s, 2H), 6.23 (d, J = 1.1 Hz, 1H), 7.47 (s, 1H).

30 STEP 2: 5'-[4-(Bis-2-chloroethyl)amino-1-butylaminomethyl]-4,4',8-trimethylpsoralen

Thionyl chloride (0.020 mL, 0.278 mmol) was added dropwise to an ice bath chilled solution of 5'-[3-(bis-2-hydroxyethyl)amino-1-butylaminomethyl]-4,4',8-trimethylpsoralen (11.6 mg, 0.0278 mmol) and pyridine (0.023 mL, 0.278 mmol) in CH_2Cl_2 (5 mL). The reaction mix was covered with a serum cap and allowed to stir overnight at room temperature, then stripped under reduced

vacuum and partitioned between CH_2Cl_2 and 10 % NaHCO_3 . The organic layer was washed several times with aqueous NaHCO_3 , then brine. After drying with anhydrous Na_2SO_4 , solvent was rotovapped off to give 5'-(4-(bis-2-chloroethyl)amino-1-butylaminomethyl]-4,4',8-trimethylpsoralen (9.3 mg, 73.8 % yield), as a yellow solid. ^1H NMR (CDCl_3): δ 1.54 (m, 4H), 2.28 (s, 3H), 2.40-2.65 (m, 8H), 2.71 (t, J = 6.8 Hz, 2H), 2.84 (t, J = 7.0 Hz, 4H), 3.48 (t, J = 7.0 Hz, 4 Hz), 3.97 (s, 2H), 6.24 (d, J = 1.1 Hz, 1H), 7.47 (s, 1H).

5'-(5-(Bis-2-chloroethyl)amino-1-pentylaminomethyl]-4,4',8-trimethylpsoralen, (compound 17)

In the same manner as the foregoing, but using N,N-bis(2-hydroxyethyl)-1,5-pentanediamine in Step 1 in place of N,N-bis(2-hydroxyethyl)-1,4-butanediamine the title compound is produced.

15 5'-(6-(Bis-2-chloroethyl)amino-1-hexylaminomethyl]-4,4',8-trimethylpsoralen,(compound 18)

In the same manner as the foregoing, but using N,N-bis(2-hydroxyethyl)-1,6-hexanediamine in Step 1 in place of N,N-bis(2-hydroxyethyl)-1,4-butanediamine the title compound is produced.

20 4'-(3-(Bis-2-chloroethyl)amino-1-propylaminomethyl]-4,5',8-trimethylpsoralen, (compound 11)

In the same manner as the foregoing, but using 4'-bromomethyl-4,5',8-trimethylpsoralen (US Patent 4,124,598) and N,N-bis(2-hydroxyethyl)-1,3-propanediamine in Step 1 in place of 5'-bromomethyl-4,4',8-trimethylpsoralen and N,N-bis(2-hydroxyethyl)-1,4-butanediamine respectively, the title compound is produced.

30 4'-(4-(Bis-2-chloroethyl)amino-1-butylaminomethyl]-4,5',8-trimethylpsoralen, (compound 12)

In the same manner as the foregoing, but using 4'-bromomethyl-4,5',8-trimethylpsoralen and N,N-bis(2-hydroxyethyl)-1,4-butanediamine in Step 1 in place of 5'-bromomethyl-4,4',8-trimethylpsoralen, the title compound is produced.

4'-(3-(Bis-2-chloroethyl)amino-1-hexylaminomethyl]-4,5',8-trimethylpsoralen,
(compound 13)

In the same manner as the foregoing, but using 4'-bromomethyl-4,5',8-trimethylpsoralen and N,N-bis(2-hydroxyethyl)-1,6-hexanediamine in Step 1 in
5 place of 5'-bromomethyl-4,4',8-trimethylpsoralen and N,N-bis(2-hydroxyethyl)-1,4-butanediamine respectively, the title compound is produced.

EXAMPLE 22

10 This example describes the synthesis of 4'-(4-(Bis-2-chloroethyl)aminobutoxy)methyl-4,5',8-trimethylpsoralen, (compound 9)

STEP 1: 4'-(4-Methanesulfonyl)-butoxy)methyl-4,5',8-trimethylpsoralen

15 A solution of 4'-(4-hydroxy)-butoxy)methyl-4,5',8-trimethylpsoralen (1-U.S. Patent 4,269,852, 91.5 mg, 0.301 mmol) in CH₂Cl₂ (5 mL) was chilled with an ice/water bath. Triethylamine (0.14 mL, 1.00 mmol) then methanesulfonyl chloride (0.070 mL, 0.903 mmol) were added dropwise. The solution was allowed to warm to room temperature and stirred overnight. The reaction
20 mixture was partitioned between CH₂Cl₂ and water. The organic layer was washed several times with aqueous NaHCO₃, then brine, and dried with anhydrous Na₂SO₄ and concentrated to give crude 4'-(4-methanesulfonyl)-butoxy)methyl-4,5',8-trimethylpsoralen (0.106 g, 86.2 % crude yield). ¹H NMR (CDCl₃): δ 1.71-1.86 (m, 4H), 2.47 (s, 6H), 2.52 (s, 3H), 2.95 (s, 3H), 3.51 (t, J = 6.0, 2H), 4.21 (t, J = 6.2, 2H), 4.59 (s, 2H), 6.20 (s, 1H), 7.54 (s, 1H).

STEP 2: 4'-(4-(Bis-2-hydroxyethyl)aminobutoxy)methyl-4,5',8-trimethylpsoralen

30 A solution of crude 4'-(4-methanesulfonyl)-butoxy)methyl-4,5',8-trimethylpsoralen (106 mg, 0.260 mmol) and diethanolamine (300 mg, 2.85 mmol) were refluxed in acetonitrile (8 mL) overnight. After rotovapping off the solvent, the resulting syrup was dissolved in CH₂Cl₂, washed several times with aqueous NaHCO₃, then brine, and dried with anhydrous Na₂SO₄ and concentrated to give crude product which was chromatographed by TLC (silica
35 gel, 95/5 CHCl₃-MeOH) to give a 4'-(4-(bis-2-hydroxyethyl)aminobutoxy)methyl-4,5',8-trimethylpsoralen as a yellow solid (32 mg, 27.1 % yield). ¹H NMR (CDCl₃):

d 1.49-1.75 (m, 4H), 2.40-2.70 (m, 15H), 3.48 (t, J = 5.8, 2H), 3.59 (t, J = 5.3, 2H), 4.61 (s, 2H), 6.24 (d, J = 1.1 Hz, 1H), 7.60 (s, 1H).

5 STEP 3: 4'-[4-(Bis-2-chloroethyl)aminobutoxy]methyl-4,5',8-trimethylpsoralen, (compound 9)

Thionyl chloride (0.040 mL, 0.55 mmol) was added dropwise to an ice bath chilled solution of 4'-[4-(bis-2-hydroxyethyl)aminobutyloxy]methyl-4,5',8-trimethylpsoralen (24.0 mg, 0.0575 mmol) and pyridine (0.050 mL, 0.62 mmol) in CH_2Cl_2 (3 mL). The reaction mix was covered with a serum cap and allowed to 10 stir overnight at room temperature, then stripped under reduced vacuum and partitioned between CH_2Cl_2 and 10 % NaHCO_3 . The organic layer was washed several times with aqueous NaHCO_3 , then brine. After drying with anhydrous Na_2SO_4 , solvent was rotovapped off to give 4'-[4-(bis-2-chloroethyl)aminobutoxy]methyl-4,5',8-trimethylpsoralen (20.5 mg, 78.5 % yield), 15 as a yellow solid. ^1H NMR (CDCl_3): d 1.40-1.74 (m, 4H), 2.45-2.65 (m, 11H), 2.81 (t, J = 7.0 Hz, 4H), 3.37-3.52 (m, 6H), 4.61 (s, 2H), 6.24 (s, 1H), 7.59 (s, 1H).

EXAMPLE 23

20 Example 23 presents compounds of the present invention that have been synthesized in a manner similar to that described in the above examples, where the nucleic acid binding ligand comprises a psoralen. The compound structures are listed in Tables 24 - 27. Examples of inactivation activity are also included in the tables for most of the compounds.

TABLE 24

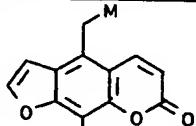
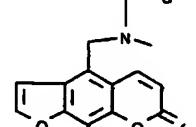
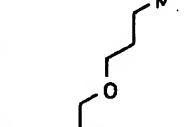
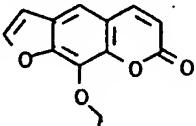
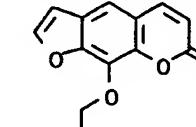
Compound	Dose for 5 log kill (μ M)	Logs Virus Killed at Fixed Drug Dose			
	R-17	R-17 0.5 μ M	R-17 2 μ M	R-17 5 μ M	CA HIV 15 μ M
M = N(CH ₂ CH ₂ Cl) ₂ NT = not tested					
QM	\leq 0.5	>6	>6	>6	2.8-4.2
 compound 2	>30	<0.5	<0.5	0.5	NT
 compound 3	>75	<0.5	<0.5	<0.5	NT
 compound 4	12-25	<0.5	0.5	2-3	NT
 compound 5	10	<0.5	<0.5	2-3	NT
 compound 6	>30	<0.5	<0.5	1	NT

TABLE 25

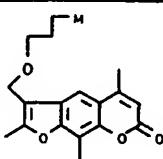
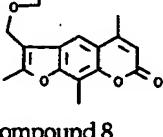
Compound	Dose for 5 log kill (μ M)	Logs Virus Killed at Fixed Drug Dose			
		R-17 0.5 μ M	R-17 2 μ M	R-17 5 μ M	CA- HIV 15 μ M
M = N(CH ₂ CH ₂ Cl) ₂ NT = not tested	R-17				
 compound 7	2	2	5	>6	0
 compound 8	7	NT	3	4	0.2

TABLE 26

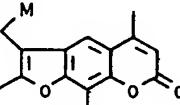
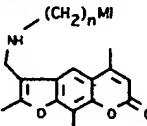
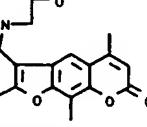
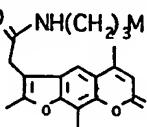
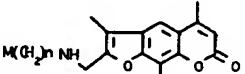
Compound	Dose for 5 log kill (μ M)	Logs Virus Killed at Fixed Drug Dose			
M = N(CH ₂ CH ₂ Cl) ₂ NT = not tested	R-17	R-17 0.5 μ M	R-17 2 μ M	R-17 5 μ M	CA- HIV 15 μ M
 compound 10	>30	NT	0.5	NT	NT
 n=3 compound 11	≤ 2	1	6	>6	0.4
n=4 compound 12	NT	NT	NT	NT	0.8
n=6 compound 13	≤ 2	NT	>6	>6	1.5
 compound 14	NT	NT	NT	NT	1.0
 n=3 compound 15	NT	NT	NT	NT	0.3

TABLE 27

Compound	Dose for 5 log kill (μ M)	Logs Virus Killed at Fixed Drug Dose			
M = N(CH₂CH₂Cl)₂ NT = not tested	R-17	R-17 0.5 μ M	R-17 2 μ M	R-17 5 μ M	CA-HIV 15 μ M
 n=4 compound 16	≤2	NT	>6	>6	0.7
n=5 compound 17	NT	NT	NT	NT	NT
n=6 compound 18	NT	NT	NT	NT	NT

5 It is to be understood that the present invention is not to be limited to the exact details of operation or exact compounds, compositions, methods, or procedures shown and described, as modifications and equivalents will be apparent to one skilled in the art. All patents described are hereby incorporated by reference.

CLAIMS

We claim:

1. A method of inactivating pathogens in a blood product, comprising:
 - 5 a) adding a compound having a mustard group and a nucleic acid binding ligand, selected from the group comprising a psoralen group and an acridine group, to a blood product suspected of containing pathogens and mixing to create a mixture, said compound reaching a final concentration sufficient to inactivate substantially all of said pathogens, and
 - 10 b) incubating said mixture without significant damage to said blood product, to create an incubated mixture.
2. The method of Claim 1, wherein said compound is added to said blood product to a final concentration of said compound of between 1 μ g/ml and 15 250 μ g/ml.
3. The method of Claim 1, wherein said mixture is incubated for between 1 minute and 48 hours.
- 20 4. The method of Claim 1, wherein said mixture is incubated for between approximately 12 and 24 hours.
5. The method of Claim 1, wherein when said compound is added to said blood product, said compound is in a mixture comprising dextrose, sodium 25 chloride, mannitol, adenine and H₂O.
6. The method of Claim 1, further comprising: c) transfusing said incubated mixture into a mammal.
- 30 7. The method of Claim 1, wherein said blood product comprises red blood cells.
8. The method of Claim 1, further comprising c) washing said blood product to remove compound from said incubated mixture.

9. The method of Claim 1, wherein said pathogens comprise viral pathogens.

10. The method of Claim 1, wherein said pathogens comprise bacterial 5 pathogens.

11. The method of Claim 1, wherein said compound is selected from the group consisting of: 8-[3-(Bis-2-chloroethyl) amino]propyloxypsoralen, 5-[3-(Bis-2-chloroethyl) aminopropoxy]methyl-8-methoxypsoralen, 5-[3-(Bis-2-10 chloroethyl) aminopropoxy]methyl-8-methoxypsoralen, 4'-[4-(Bis-2-chloroethyl)aminobutoxy]methyl-4,5',8-trimethylpsoralen, and N1,N1-bis (2-chloroethyl)-N4-(6-chloro-2-methoxy-9-acridinyl)-1,4-pentanediamine.

12. The method of Claim 1, wherein more than one of said compounds 15 is added to said blood product.

13. The method of Claim 1, further comprising: removing said compound from said incubated mixture with an adsorbent material.

20 14. A method of inactivating pathogens in a blood product, comprising:
a) adding a compound having a mustard group and a nucleic acid binding ligand, selected from the group consisting of a psoralen group and an acridine group, to a blood product comprising red blood cells suspected of containing pathogens and mixing to create a mixture, said compound reaching a 25 final concentration sufficient to inactivate substantially all of said pathogens, and
b) incubating said mixture for between 1 minute and 48 hours, without significant damage to said red blood cells, to create an incubated mixture.

30 15. The method of Claim 14, wherein said compound is added to said blood product to a final concentration of said compound of between 1 μ g/ml and 250 μ g/ml.

35 16. The method of Claim 14, wherein when said compound is added to said blood product, said compound is in a solution comprising dextrose, sodium chloride, mannitol, adenine and H₂O.

17. The method of Claim 14, further comprising: c) transfusing said incubated mixture into a mammal.

18. The method of Claim 14, wherein said pathogens comprise viral
5 pathogens.

19. The method of Claim 14, wherein said pathogens comprise bacterial pathogens.

10 20. The method of Claim 14, further comprising, (c) washing said blood product to remove compound from said incubated mixture.

15 21. The method of Claim 14, wherein said compound is selected from the group consisting of: 8-[3-(Bis-2-chloroethyl) amino]propyloxysoralen, 5-[3-(Bis-2-chloroethyl) aminopropoxy]methyl-8-methoxysoralen, 5-[3-(Bis-2-chloroethyl) aminopropoxy]methyl-8-methoxysoralen, 4'-[4-(Bis-2-chloroethyl)aminobutoxy]methyl-4,5',8-trimethylpsoralen, and N1,N1-bis (2-chloroethyl)-N4-(6-chloro-2-methoxy-9-acridinyl)-1,4-pentanediamine.

20 22. The method of Claim 14, wherein more than one of said compounds is added to said blood product.

25 23. The method of Claim 14, further comprising: c) after incubating said mixture, removing said compound from said incubated mixture with an adsorbent material.

24. An improved method of treating biological compositions, comprising:

30 a) adding a compound having a nucleic acid binding ligand and a mustard group to a biological composition and mixing to create a mixture, and
b) incubating said mixture to create an incubated mixture.

35 25. The method of Claim 24, wherein said compound is added to said biological composition to a final concentration of said compound of between 1 μ g/ml and 250 μ g/ml.

26. The method of Claim 24, wherein said mixture is incubated for between 1 minute and 48 hours.

27. The method of Claim 24, wherein when said compound is added to 5 said biological composition, said compound is in a mixture comprising dextrose, sodium chloride, mannitol, adenine and H₂O.

28. The method of Claim 24, wherein said biological composition comprises a blood product.

10

29. The method of Claim 28, further comprising: c) transfusing said incubated mixture into a mammal.

15

30. The method of Claim 28, wherein said blood product comprises red blood cells.

31. The method of Claim 24, further comprising, (c) washing said blood product to remove said compound from said incubated mixture.

20

32. The method of Claim 24, wherein said blood product further comprises viral pathogens.

33. The method of Claim 24, wherein said blood product further comprises bacterial pathogens.

25

34. The method of Claim 24, wherein said compound is selected from the group consisting of: 8-[3-(Bis-2-chloroethyl) amino]propyloxysoralen, 5-[3-(Bis-2-chloroethyl) aminopropoxy]methyl-8-methoxysoralen, 5-[3-(Bis-2-chloroethyl) aminopropoxy]methyl-8-methoxysoralen, 4'-[4-(Bis-2-chloroethyl)aminobutoxy]methyl-4,5',8-trimethylpsoralen, and N1,N1-bis (2-chloroethyl)-N4-(6-chloro-2-methoxy-9-acridinyl)-1,4-pentanediamine.

35. The method of Claim 24, further comprising: removing said compound from said incubated mixture with an adsorbent material.

35

36. An improved method of treating clinical samples, comprising, in the following order:

a) providing: (1) a compound having a mustard group and a nucleic acid binding ligand, selected from the group consisting of a psoralen

5 group and an acridine group, and (2) a clinical sample intended for in vitro clinical testing;

b) adding said compound to said clinical sample and mixing to create a mixture,

c) incubating said mixture for between 1 minute and 48 hours, and

10 d) measuring the level of a clinical chemistry analyte in said clinical sample.

37. The method of Claim 36, wherein said compound is selected from the group consisting of: 8-[3-(Bis-2-chloroethyl) amino]propyloxypsoralen, 5-[3-(Bis-2-chloroethyl) aminopropoxy]methyl-8-methoxypsoralen, 5-[3-(Bis-2-chloroethyl) aminopropoxy]methyl-8-methoxypsoralen, 4'-[4-(Bis-2-chloroethyl)aminobutoxy]methyl-4,5',8-trimethylpsoralen, and N1,N1-bis (2-chloroethyl)-N4-(6-chloro-2-methoxy-9-acridinyl)-1,4-pentanediamine.

20 38. The method of Claim 36, wherein said clinical sample comprises red blood cells.

39. The method of Claim 38, wherein said red blood cells further comprise viral pathogens.

25

40. The method of Claim 38, wherein said red blood cells further comprise bacterial pathogens.

41. The method of Claim 36, wherein step c) is performed without significant damage to said clinical chemistry analog.

30 42 A method of inactivating pathogens in a red blood cell containing composition, comprising:

35 a) adding a compound having a nucleic acid binding ligand and a mustard group to a blood product comprising red blood cells, where said blood product is suspected of containing pathogens, to create a mixture, said compound

reaching a final concentration sufficient to inactivate substantially all of said pathogens,

5 b) incubating said mixture *in vitro* for between 1 minute and 48 hours while retaining the ability of said blood product to function for the purpose said blood product was prepared, to create an incubated mixture, and

 c) transfusing said incubated mixture into a mammal.

10 43. The method of Claim 42, wherein said compound is added to said blood product to a final concentration of said compound of between 1 μ g/ml and 250 μ g/ml.

15 44. The method of Claim 42, wherein when said compound having a nucleic acid binding ligand and a mustard group is added to said blood product, said compound is in a solution comprising dextrose, sodium chloride, mannitol, adenine and H₂O.

20 45. The method of Claim 42, wherein said pathogens comprise viral pathogens.

25 46. The method of Claim 42, wherein said pathogens comprise bacterial pathogens.

30 47. The method of Claim 42, wherein said compound is selected from the group consisting of: 8-[3-(Bis-2-chloroethyl) amino]propyloxysoralen, 5-[3-(Bis-2-chloroethyl) aminopropoxy]methyl-8-methoxysoralen, 5-[3-(Bis-2-chloroethyl) aminopropoxy]methyl-8-methoxysoralen, 4'-[4-(Bis-2-chloroethyl)aminobutoxy]methyl-4,5',8-trimethylpsoralen, and N1,N1-bis (2-chloroethyl)-N4-(6-chloro-2-methoxy-9-acridinyl)-1,4-pentanediamine.

35 48. The method of Claim 42, wherein more than one of said compounds is added to said blood product.

49. The method of Claim 42, further comprising: removing said compound from said incubated mixture with an adsorbent material.

50. The method of Claim 42, wherein said nucleic acid binding ligand of said compound is selected from the group comprising: a psoralen group and a 9-aminoacridine group.

5 51. A method of inactivating pathogens in a red blood cell containing composition, comprising:

10 a) adding a compound having an acridine group and a mustard group to a blood product comprising red blood cells, where said blood product is suspected of containing pathogens, to create a mixture, said compound reaching a final concentration sufficient to inactivate substantially all of said pathogens, and

15 b) incubating said mixture in vitro for between 1 minute and 48 hours, while retaining the ability of said blood product to function for the purpose said blood product was prepared, to create an incubated mixture.

15

52. The method of Claim 51, wherein said compound is added to said blood product comprising red blood cells to a final concentration of between 1 μ g/ml and 250 μ g/ml.

20

53. The method of Claim 51, wherein when said compound is added to said blood product comprising red blood cells, said mixture further comprises dextrose, sodium chloride, mannitol, adenine and H₂O.

25

54. The method of Claim 51, further comprising: c) transfusing said mixture into a mammal.

55. The method of Claim 51, wherein said pathogens comprise viral pathogens.

30

56. The method of Claim 51, wherein said pathogens comprise bacterial pathogens.

57. The method of Claim 51, wherein said compound is N1,N1-bis (2-chloroethyl)-N4-(6-chloro-2-methoxy-9-acridinyl)-1,4-pentanediamine.

35

58. The method of Claim 51, further comprising: removing said compound from said incubated mixture with an adsorbent material.

59. The method of Claim 51, further comprising mixing said mixture during incubation.

60. A composition of matter, comprising: 8-[3-(Bis-2-chloroethyl)amino]propyloxypsoralen.

10 61. A composition of matter, comprising: 5-[3-(Bis-2-chloroethyl)aminopropoxy]methyl-8-methoxypsoralen.

15 62. A composition of matter, comprising: 5-[3-(Bis-2-chloroethyl)aminopropoxy]methyl-8-methoxypsoralen.

63. A composition of matter, comprising: 4'-[4-(Bis-2-chloroethyl)aminobutoxy]methyl-4,5',8-trimethylpsoralen.

20 64. A composition of matter, comprising: 5-[N,N-bis(2-chloroethyl)amino]methyl-8-methoxypsoralen.

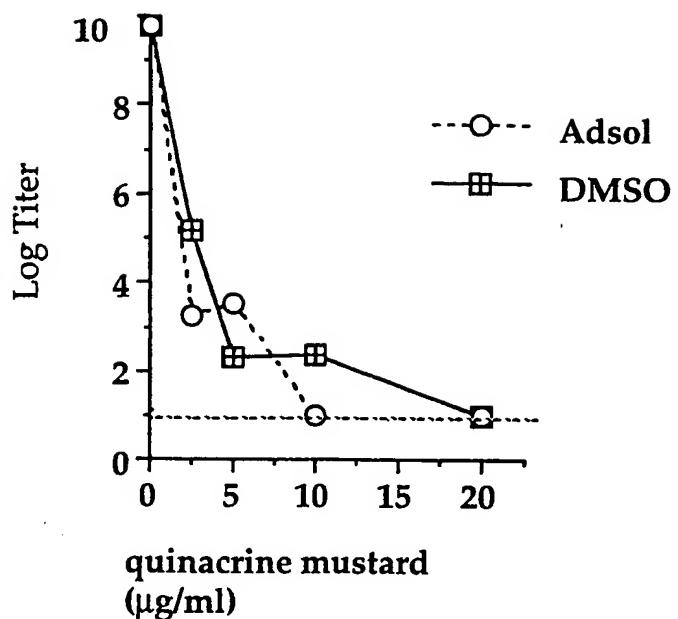


FIG. 1

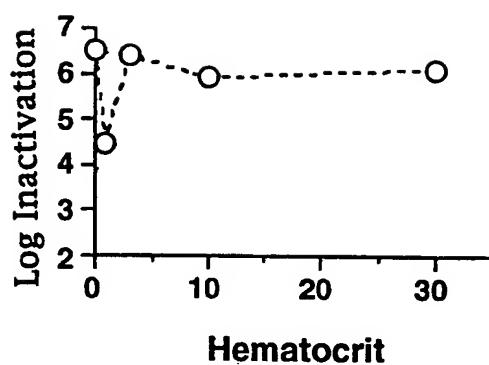


FIG. 2

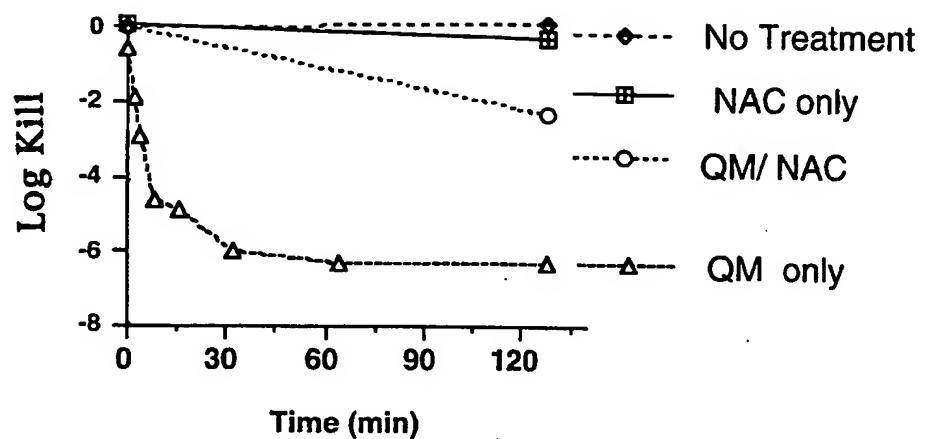


FIG. 3

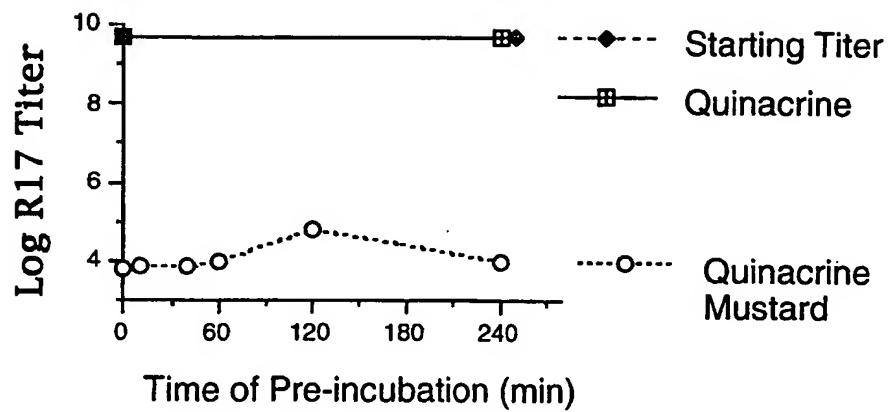


FIG. 4

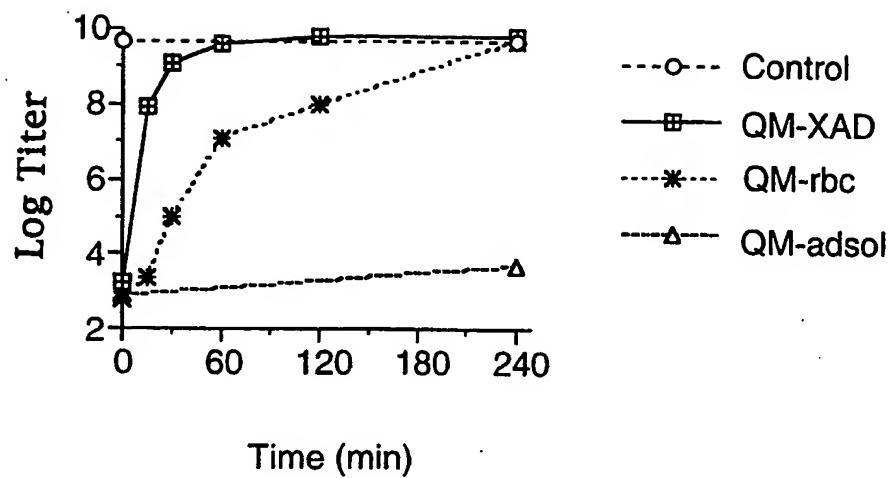


FIG. 5

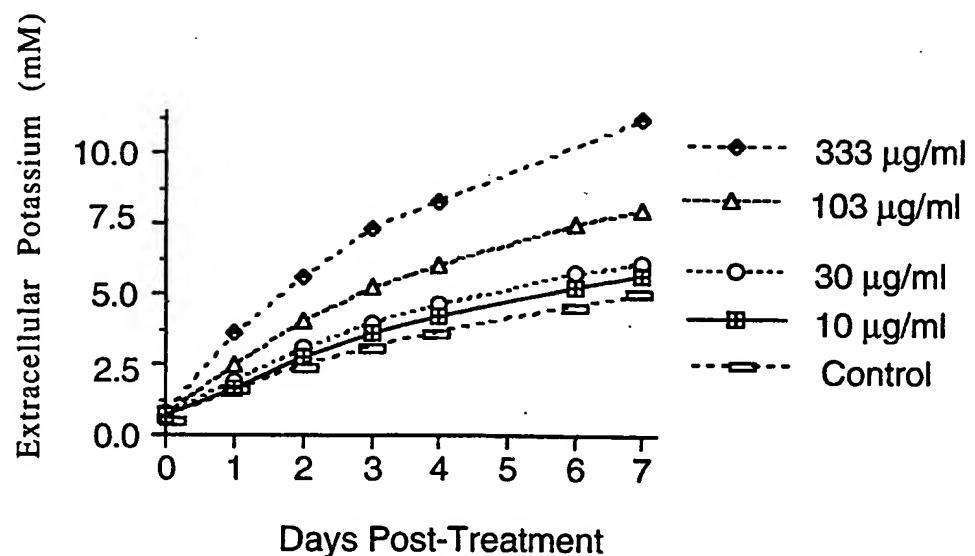


FIG. 6

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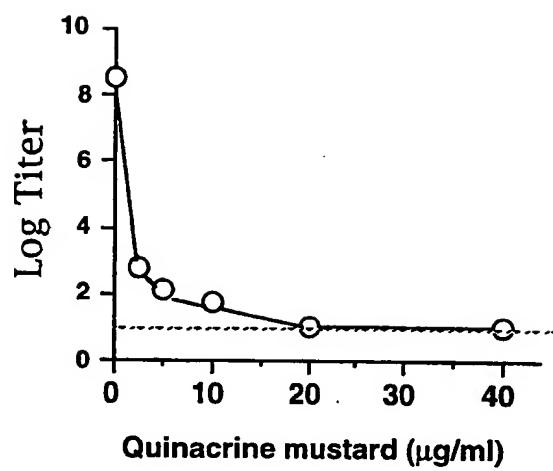


FIG. 7

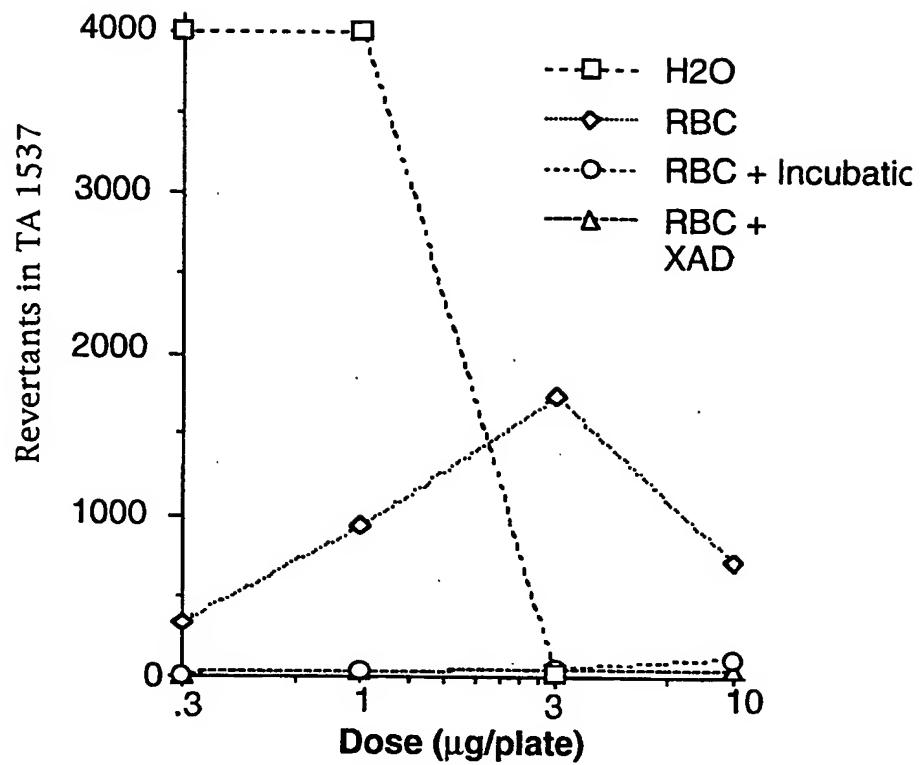


FIG. 8

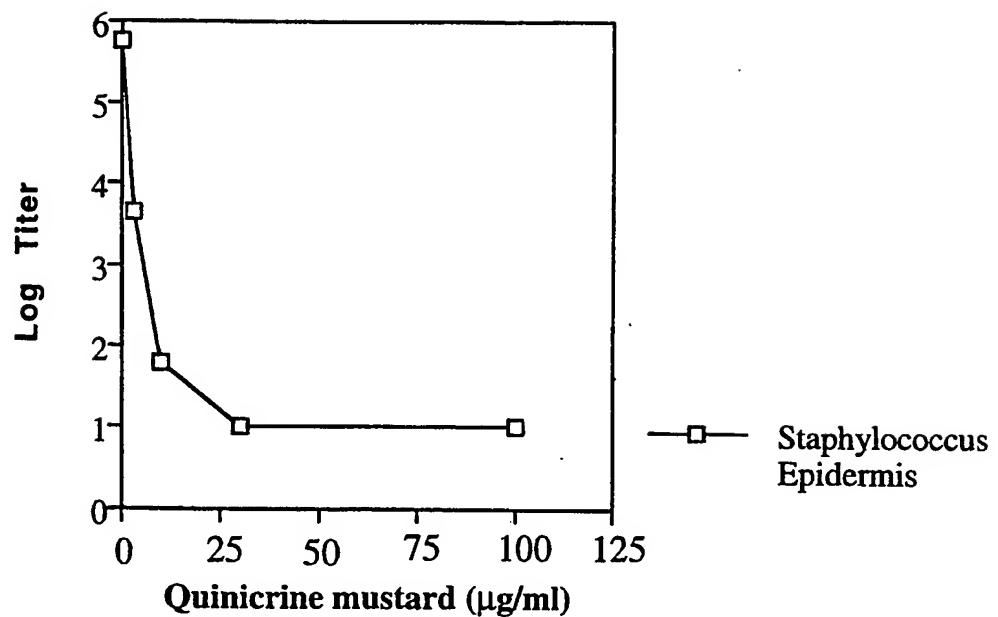


FIG. 9

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/09616

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A01N 1/02; C12N 7/06

US CL :435/2, 238

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/2, 238

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, CAS-ONLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	LOGRIppo et al. 'Chemical and Combined Methods for Plasma Sterilization.' In: Proceedings of the Sixth Congress of the International Society of Blood Transfusion. Edited by L. Hollander. Basel: Bibliotheca Haematologica, 1958, Vol. 7, pages 225-230, see entire document.	1-59
X	MATTES et al. GC-rich regions in genomes as targets for DNA alkylation. Carcinogenesis. 1988, Vol. 9, No. 11, pages 2065-2072, especially abstract.	24-26, 34, 36, 37, 41
---		-----
Y	US 4,182,750 A (SULLIVAN ET AL.) 08 January 1980 (08.01.80), see abstract.	1-25, 27-33, 35, 38-40, 42-59
Y		13, 23, 35, 49, 58

Further documents are listed in the continuation of Box C.

See patent family annex.

•	Special categories of cited documents:	
"A"	document defining the general state of the art which is not considered to be of particular relevance	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"E"	earlier document published on or after the international filing date	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means	"&" document member of the same patent family
"P"	document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

23 AUGUST 1996

Date of mailing of the international search report

09 SEP 1996

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US96/09616

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:

2. Claims Nos.: because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

The additional search fees were accompanied by the applicant's protest.

No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/09616

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claims 1-60, drawn to methods of using a compound which has a mustard group and an acridine or psoralen group to inactivate pathogens and first specific embodiment of the compound, 8-[3-(Bis-2-chloroethyl)amino]propyloxysorafen.

Group II, claims 61 and 62, drawn to a second compound, 5-[3-(Bis-2-chloroethyl)amino]propyloxy)methyl-8-methoxysorafen.

Group III, claim 63, drawn to a third compound, 4'-(4-(Bis-2-chloroethyl)amino)butoxy)methyl-4,5',8-trimethylpsoralen.

Group IV, claim 64, drawn to a fourth compound, 5-[N,N-bis(2-chloroethyl)amino]methyl-8-methoxysorafen.

The inventions listed as Groups I-IV do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: the inventions do not match a permitted category as Rule 13.2 does not provide for multiple compositions which do not share the same of corresponding special technical feature in one category (See 37 CFR 1.475(d)).